Intratumor Microvessel Density Assessment. Immunohistochemical staining for CD31 and microvessel counting of CD31-positive vessels were performed as described previously (11).

Analyses of Tumors Administered with FI-Labeled siRNA. FI-labeled VEGF siRNA no. 3-SCR (VEGF siRNA-FI; 55.2 μg/tumor) with atelocollagen was directly injected into PC-3 tumor (tumor volume: ~100 mm^3) in the same manner as described in the above section. After 1 and 8 days, tumors were removed and snap frozen in liquid nitrogen and then 2-μm thick sections were cut with cryostat and observed under a confocal microscope system (MRC 1024; Bio-Rad). Various amounts of the VEGF siRNA-FI (10, 5, and 1 μM) with atelocollagen were also injected. After 1 and 8 days, excised tumors were homogenized in ice-cold CelLytic-MT Mammalian Tissue Lysis Extraction Reagent (1 ml) with protease inhibitor mixture (Sigma Chemical Co., St Louis, MO) and then centrifuged. The fluorescence intensity in each supernatant was measured by a Shimadzu Spectrofluorophotometer (Model RF-5000). The wavelengths for excitation and emission were 490 and 530 nm, respectively. Each fluorescence intensity value was corrected by a protein concentration.

PAGE Analysis of ^{32}P-Labeled siRNA Injected into the Tumors. ^{32}P-labeled siRNA with atelocollagen (^{32}P-labeled siRNA, 10 μM; atelocollagen, 1.75%) was injected into PC-3 tumors. On the next day and 2 and 7 days after the injection, each tumor was excised and homogenized in 0.5 ml of ice-cold 10 mM Tris-HCl (pH 8.0) with 0.1% Triton X-100, 50 mM NaCl, and RNase inhibitor (1 units/μl, Roche) and then centrifuged. Proteinase K (1 mg; Roche) was directly added to the supernatant and incubated for 6 h at 37°C and centrifuged. The supernatant was analyzed by PAGE (15% gel) followed by autoradiography.

Statistical Analysis. The data were analyzed using the Mann-Whitney *U* test, and Ps < 0.05 were considered to indicate significant differences.

Results

Effects of VEGF siRNAs on the Expression of VEGF from PC-3 Cells. We examined five VEGF siRNAs to target human VEGF as described in Fig. 1*A*. Of the five siRNAs synthesized, VEGF siRNA no. 3 potently suppressed the synthesis and secretion of VEGF in human prostate carcinoma cells, PC-3, after transfection with LipofectAMINE-PLUS (Fig. 2*A*). Scrambled siRNA (VEGF siRNA no. 3-SCR) showed no effects (Fig. 2*B*). ELISA for VEGF revealed that VEGF siRNA no. 3 (100 nM) suppressed VEGF production to 1.3–1.6% of that in the control cultures as shown in Fig. 2, *A* and *B*. On the basis of these results, we selected VEGF siRNA no. 3 as the most highly functional. To investigate the specificity of the RNAi system, we made mutant siRNAs containing either one or two central mismatches (Fig. 1*B*). We found that the single and double mutants lost their RNAi activity (Fig. 2*B*). VEGF siRNA no. 3 decreased the VEGF mRNA level compared with VEGF siRNA no. 3-SCR or untreated cultures (Fig. 2*C*), whereas the expression level of VEGF-B, VEGF-C, VEGF-D, and placenta growth factor did not change upon transfection of VEGF siRNA no. 3 (Fig. 2*D*). We also investigated cellular uptake of siRNA after transfection. After uptake of VEGF siRNA-FI by PC-3 cells, a characteristic spotty distribution around the nuclei was observed (Fig. 2*E*). The efficiency for transfection (percentage) to the cells using ^{32}P-labeled siRNA was estimated as 85.2 ± 1.2% (n = 3 dishes).

Treatment of the Established PC-3 Xenograft by VEGF siRNA. PC-3 cells (3.0 × 10^6) were injected s.c. into the flank of nude mice. By 3 weeks, visible tumors had developed at the injection sites (mean tumor volume, 54.2 mm^3; n = 30). To determine the therapeutic effectiveness of VEGF siRNA, intratumoral treatment with VEGF siRNAs with atelocollagen or atelocollagen alone was started and repeated every 10 days for a total of four times. As shown in Fig. 3, *A* and *B*, VEGF siRNA no. 3 markedly suppressed tumor growth compared with VEGF siRNA no. 3-SCR or atelocollagen alone ($P < 0.001$). These growth inhibitory effects were dependent on the dose of the VEGF siRNA no. 3 (Fig. 3*A*). Furthermore, we also found that the decrease of the VEGF contents in tumors treated with VEGF siRNA no. 3 was correlated with the injected doses of VEGF siRNA no. 3 (Fig. 3*C*). No gross adverse effects, *i.e.*, the loss of body weight, were observed during the experimental periods (data not shown).

Tumor Vascularity *in Vivo*. To assess the relationship between the therapeutic effects by VEGF siRNA and tumor-associated neovascularization, we stained intratumoral vessels with CD31/PECAM-

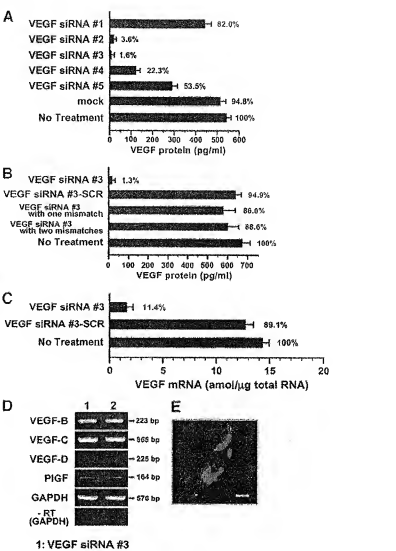

Fig. 2. Decrease in secreted vascular endothelial growth factor (VEGF) and the mRNA levels in PC-3 cells transfected with various small interfering RNAs (siRNAs). *A*, cells were transfected with VEGF siRNA nos. 1, 2, 3, 4, and 5, LipofectAMINE-PLUS alone (mock), or no siRNA, respectively. *B*, cells were transfected with VEGF siRNA no. 3, VEGF siRNA no. 3-SCR, VEGF siRNA no. 3 with one mismatch, VEGF siRNA no. 3 with two mismatches, or no siRNA, respectively. In both *A* and *B*, VEGF concentration in the conditioned media was determined by ELISA for human VEGF. Each *bar* represents the mean ± SD (n = 4 dishes). *C*, decreased VEGF mRNA levels of PC-3 cells transfected with various siRNAs. VEGF mRNA levels were determined with a Quantikine mRNA colorimetric quantification kit. Each *bar* represents the mean ± SD (n = 3 dishes). *D*, the expression of VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) mRNA in the cells transfected with VEGF siRNA no. 3 or VEGF siRNA no. 3-SCR. Total RNA was extracted from the cells and processed for reverse transcription-PCR (RT-PCR). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a control. *Lane 1*, VEGF siRNA no. 3; *Lane 2*, VEGF siRNA no. 3-SCR. Predicted size of each PCR product was shown in the figure. −RT, no reverse transcriptase. *E*, cellular uptake of VEGF siRNA-FI. PC-3 cells were transfected with VEGF siRNA-fluorescein for 4 h, washed with PBS, fixed in 4% paraformaldehyde, mounted with a ProLong Antifade kit, and subjected to confocal microscopy. *Bar*, 10 μm.

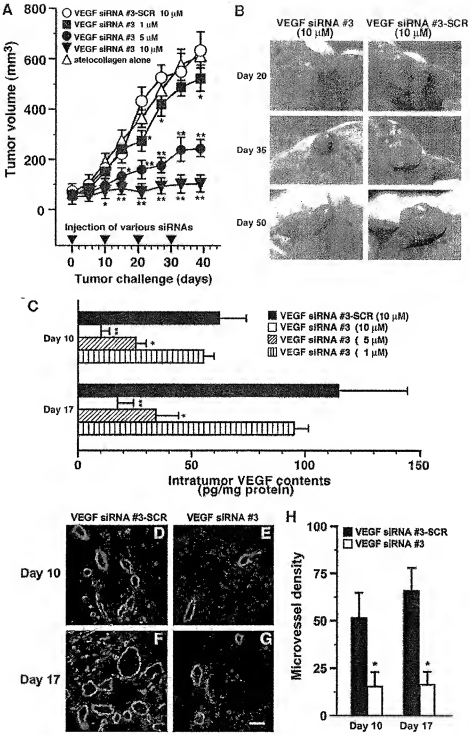

Fig. 3. Antitumor effect of vascular endothelial growth factor (VEGF) small interfering RNA (siRNA) in the PC-3 xenograft. *A*, tumor growth curves. On days 0, 10, 20, and 30, VEGF siRNA no. 3 (■, 1 μM; ●, 5 μM; ▲, 10 μM), VEGF siRNA no. 3-SCR (○, 10 μM), or PBS (△) was mixed with atelocollagen, and 50 μl of each mixture were injected into the tumor region, as indicated in the figure. Day 0 corresponds to 3 weeks after inoculation of cells when the tumor volume was ~50–60 mm^3. Tumor diameters were measured at a regular interval for up to 40 days with calipers, and the tumor volume was calculated. Results represent the means ± SE (n = 6 tumors). *, $P < 0.05$; **, $P < 0.001$ *versus* VEGF siRNA no. 3-SCR 10 μM. *B*, photos of PC-3 xenografts. During VEGF siRNA treatment (concentration of siRNA: 10 μM), PC-3 tumors were photographed. *C*, VEGF contents in tumors. Experiments were performed as in *A*. The excised tumors (on days 10 and 17) were homogenized in ice-cold CelLytic-MT Mammalian Tissue Lysis/Extraction Reagent with protease inhibitor mixture (Sigma Chemical Co.) and then centrifuged. The amount of VEGF in each supernatant was measured by ELISA. **, $P < 0.001$; *, $P < 0.01$ *versus* VEGF siRNA no. 3-SCR (10 μM). Results represent the mean ± SD (n = 4 tumors). *D–H*, decreased vessel density in tumors treated with VEGF siRNA. Histological sections from tumors injected with VEGF siRNA no. 3 or VEGF siRNA no. 3-SCR mixed with atelocollagen were immunostained for endothelial cells with anti-CD31 antibodies. We examined four excised tumors on both day 10 and 17 after injection and the intratumoral microvessel density (vessels/mm^2) was determined. *D* and *F*, VEGF siRNA no. 3-SCR; *E* and *G*, VEGF siRNA no. 3. *Bar*, 50 μm. *H*, decreased microvessel density in tumors treated with VEGF siRNA no. 3. Results represent the mean ± SD (n = 4 tumors). *, $P < 0.001$ *versus* VEGF siRNA no. 3-SCR.

1-specific antibody. Representative results of this staining are shown in Fig. 3*D–G*, and quantitative results of the microvessel density analysis are given in Fig. 3*H*. We observed a dramatically lower microvessel density in tumors treated with VEGF siRNA no. 3 at both 10 and 17 days after the first injection, whereas the tumors treated with VEGF siRNA no. 3-SCR showed a higher microvessel density (Fig. 3*D–H*).

Effects of Atelocollagen on Stability of Injected siRNA. To verify the capability of atelocollagen to stabilize the injected siRNA in tumors, we injected FI-labeled VEGF siRNA no. 3-SCR (VEGF siRNA-FI) mixed with atelocollagen directly into PC-3 tumors. Representative images of the cryostat sections were shown in Fig. 4*A*. We observed a strong green fluorescence in tumors upon injection of VEGF siRNA-FI with atelocollagen at least up to 8 days after injection while only a weak fluorescence in VEGF siRNA-FI alone. Furthermore, fluorescence intensity in tumors treated with VEGF siRNA-FI with atelocollagen was quite higher than those of VEGF siRNA-FI alone (Fig. 4*C*). Intensities of fluorescence remained in the tumors correlated with the injected amounts of VEGF siRNA-FI mixed with atelocollagen (Fig. 4*C*). These observations suggested that

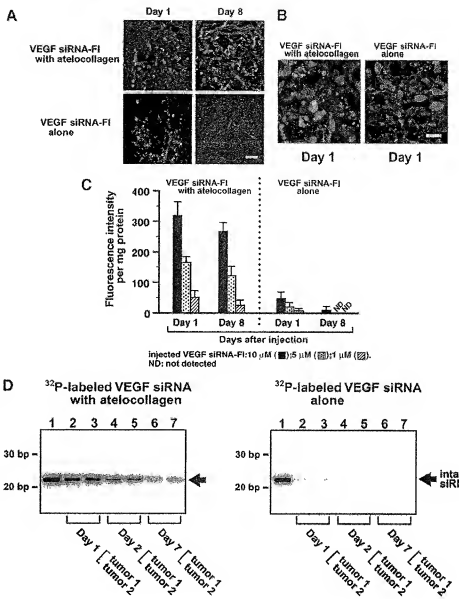

Fig. 4. Role of atelocollagen as a delivery reagent of siRNA *in vivo*. *A*, vascular endothelial growth factor (VEGF) small interfering RNA-fluorescein (siRNA-FI; 53.2 μg) with or without atelocollagen were directly injected in PC-3 tumors (~ 100 mm^3). On the next day and 8 days after injection, tumors were excised and sections were cut. Fluorescence was observed under a confocal microscope. *Bar*, 100 μm. *B*, nuclei were stained with propidium iodide (0.3 μg/ml), and the merged images with higher magnifications using oil lens were obtained. *Bar*, 20 μm. *C*, fluorescence intensity in homogenized supernatant from each tumor was measured. Each concentration of VEGF siRNA-FI (10, 5, and 1 μM) with or without atelocollagen were injected in tumors as in *A*. On the next day and 8 days after injection, tumors were excised and homogenized. ND, not detected. Results represent the mean ± SD ($n = 3$ tumors). *D*, autoradiograms of ^{32}P-labeled VEGF siRNA injected into tumors. ^{32}P-labeled VEGF siRNA with or without atelocollagen was injected into PC-3 tumors. On the next day and 2 and 7 days after injection, tumors were excised, homogenized, and digested with proteinase K. Supernatant after centrifugation was analyzed with PAGE (15% gel), and autoradiogram was obtained. *Left panel*, ^{32}P-labeled VEGF siRNA with atelocollagen; *right panel*, ^{32}P-labeled VEGF siRNA alone. *Lane 1*, intact ^{32}P-labeled VEGF siRNA; *Lanes 2* and *3*, ^{32}P-labeled VEGF siRNA from tumors on day 1; *Lanes 4* and *5*, ^{32}P-labeled VEGF siRNA on day 2; *Lanes 6* and *7*, ^{32}P-labeled VEGF siRNA on day 7. *Arrows*, intact ^{32}P-labeled VEGF siRNA (21-mer) before injection.

atelocollagen contributed to the increased stability of VEGF siRNA injected in tumors and that the injection interval (every 10 days) of VEGF siRNA upon tumor treatments (Fig. 3*A*) was appropriate. Moreover, VEGF siRNA-FI was visible inside tumor cells in the case of transfection with atelocollagen *in vivo* (yellow color) but not in the case of transfection without atelocollagen (Fig. 4*B*).

Finally, using ^{32}P-labeled siRNA, we investigated whether the siRNA injected in tumors remained intact and, if intact, how long it persisted. ^{32}P-labeled siRNA mixed with atelocollagen existed at least 7 days in tumors and remained intact (Fig. 4*D*). On the other hand, ^{32}P-labeled siRNA alone was rapidly degraded and vanished from the tumors (Fig. 4*D*).

Discussion

A siRNA to VEGF successfully inhibited the secretion and expression of VEGF in PC-3, human prostate carcinoma cells, leading to the potent suppression of tumor growth in its xenograft model. These results clearly demonstrated that a novel VEGF blockade system by RNAi is valid as a therapeutic. It is indispensable to use a suitable siRNA delivery system to obtain the maximal therapeutic effects. In this study, atelocollagen, a new gene delivery system, contributed significantly to the antitumoral therapeutic effect of VEGF siRNA. Atelocollagen, which was developed by Ochiya *et al.* (10), is soluble at a lower temperature (<10°C) but solidifies to refibrillation upon injection into an animal body (37°C). They have developed a technology in which plasmid DNA is embedded in atelocollagen by which the quantity and period of the gene expression are well controlled *in vivo* (10). Atelocollagen can protect plasmid DNA, antisense oligodeoxynucleotide, and adenoviral vectors from degradation by several nucleases, proteases, and antibodies, thereby prolonging the half-life of embedded biomaterials (10, 17). In the present study, we successfully demonstrated, by using FI- and radiolabeled siRNAs, that atelocollagen actually stabilized the siRNA injected in the tumors for at least a week (Fig. 4). Atelocollagen was, thus, crucial to extend a half-life of the siRNA dramatically and to keep it intact when embedded in the animal body. We also observed that atelocollagen was effective on the transfection of siRNAs *in vivo* (Fig. 4*B*). Furthermore, atelocollagen shows neither antigenicity nor toxicity in animals be-

cause antigenic telopeptides attached to both ends of collagen are eliminated by pepsin digestion (10). Consistently, atelocollagen caused no obvious hepatocellular or renal damage upon local injection into the tumor in nude mice (Y. Takei, unpublished data). Taken together, we conclude that atelocollagen is a superior delivery reagent of siRNAs *in vivo*.

Bergers *et al.* (18) have shown that the makeup of the tumor vasculature varies at different stages of tumor development, so the inhibitor efficacy might depend on its application during a specific phase of tumorigenesis. In fact, they observed that the VEGF receptor inhibitor SU5416 blocks the angiogenic switch and prevents the growth of premalignant tumors but does not induce regression of late-stage, well-vascularized tumors (18). This reveals the importance of VEGF signaling during the angiogenic switch and initial tumor growth but not in large tumors with an established vasculature. In light of these observations, we began the treatment with VEGF siRNA when the tumors inoculated in nude mice were still small (initial tumor volume = 50–60 mm^3) before the vasculature was well established in the tumors.

In conclusion, we demonstrated potent growth inhibitory effects of VEGF siRNA on prostate carcinoma *in vivo*, potentially applicable to the treatment of cancers as an antiangiogenic therapeutic.

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EXHIBIT 14

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Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing *in vitro* and *in vivo*

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ABSTRACT

Silencing gene expression by siRNAs is rapidly becoming a powerful tool for the genetic analysis of mammalian cells. However, the rapid degradation of siRNA and the limited duration of its action call for an efficient delivery technology. Accordingly, we describe here that Atelocollagen complexed with siRNA is resistant to nucleases and is efficiently transduced into cells, thereby allowing long-term gene silencing. Site-specific *in vivo* administration of an anti-luciferase siRNA/Atelocollagen complex reduced luciferase expression in a xenografted tumor. Furthermore, Atelocollagen-mediated transfer of siRNA *in vivo* showed efficient inhibition of tumor growth in an orthotopic xenograft model of a human non-seminomatous germ cell tumor. Thus, for clinical applications of siRNA, an Atelocollagen-based non-viral delivery method could be a reliable approach to achieve maximal function of siRNA *in vivo*.

INTRODUCTION

RNA interference (RNAi) as a protecting mechanism against invasion of foreign genes was first described in *Caenorhabditis elegans* (1) and has subsequently been demonstrated in diverse eukaryotes, such as insects, plants, fungi and vertebrates (2). In many eukaryotes, expression of nuclear-encoded mRNA can be strongly inhibited by the presence of a double-stranded RNA (dsRNA) corresponding to exon sequences in the mRNA. RNAi can be exploited in cultured mammalian cells by introducing shorter, synthetic duplex RNAs (~20 nt) through liposome transfection (3–5) and a peptide-based delivery (6). In mammalian cells, siRNAs have become a new and powerful alternative to other genetic knockdown methods for the analysis of loss-of-function phenotypes. In theory, the technique is simple and elegant. In practice, however, limited stability *in vivo* and the absence of a reliable delivery method hamper the utility of siRNA for therapeutic application. Reports have shown that liposomes (7,8), adenovirus (9), adeno-associated viral vectors (10) and lentivirus (11) can be considered as useful delivery systems. A virus vector-based siRNA delivery overcomes the problem of poor transfection efficiency of plasmid-based systems. However, viral vectors have several limitations when they are used *in vivo*.

Atelocollagen is a highly purified pepsin-treated type I collagen from calf dermis. Collagen is a fibrous protein in the connective tissue that plays an important role in the maintenance of the morphology of tissues and organs. A collagen molecule has an amino acid sequence called as telopeptide at both N- and C-terminals, which confers most of the collagen's antigenicity. Atelocollagen obtained by pepsin treatment is low in immunogenicity because it is free from telopeptides (12), and it is used clinically for a wide range of purposes, including wound-healing, vessel prosthesis and also as a bone cartilage substitute and hemostatic agent (13). We have demonstrated previously that Atelocollagen complexed with DNA molecules was efficiently transduced into mammalian cells (14) and allowed long-term gene expression (15). Since Atelocollagen allows increased cellular uptake, nuclease resistance and prolonged release of genes and oligonucleotides (13), an Atelocollagen complex is applicable for an efficient delivery of siRNA *in vitro*. Furthermore, Atelocollagen displays low-toxicity and low-immunogenicity when it is transplanted *in vivo* (13,16). Thus, our gene delivery method using an Atelocollagen implant should permit safe and efficient siRNA-mediated gene silencing in therapeutic applications.

MATERIALS AND METHODS

Atelocollagen

Atelocollagen is a highly purified type I collagen of calf dermis with pepsin treatment (Koken Co., Ltd, Tokyo, Japan).

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siRNA preparation

Synthetic 21-nt RNAs were purchased from Dharmacon (Lafayette, CO) in deprotected, desalted and annealed form. The sequence of our prepared human fibroblast growth factor (FGF)-4 (HST-1/FGF-4) siRNA was 5′-CGAUGAGUGCAC-GUUCAAGdTdT-3′; 3′-dTdTGCUACUCACGUGCAAGU-UC-5′. Non-specific control siRNA duplex (VIII), luciferase GL3 siRNA duplex and luciferase GL2 siRNA were also purchased from Dharmacon, and were used as controls.

Formation of siRNA/Atelocollagen complex

The siRNAs and Atelocollagen complexes were prepared as follows. An equal volume of Atelocollagen (in PBS at pH 7.4) and siRNAs solution was combined and mixed by rotation at 4°C for 20 min. The complex was then kept at 4°C for 16 h before use. The final concentration of Atelocollagen *in vitro* and *in vivo* was 0.008 and 0.5%, respectively.

Stability of siRNA/Atelocollagen complex

An aliquot of 0.9 μg of siRNAs (luciferase GL3 duplex) and 0.5% Atelocollagen or cationic liposome (jetSI; Polyplus-transfection SAS, Illkirch Cedex, France) complexes were incubated in the presence of 0.1 μg/μl RNase A (NipponGene, Tokyo, Japan) for 0, 5, 15, 30, 45 and 60 min at 37°C. The solutions were extracted with phenol and phenol/chloroform/isoamyl alcohol (25:24:1). The siRNAs were precipitated with ethanol and agarose gel electrophoresed (3.5%) and visualized by ethidium bromide staining.

Cell lines

NEC8 cells (American Type Culture Collection, Rockville, MD) derived from human testicular tumor were maintained in DMEM with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2. Increased expression of the *HST-1/FGF-4* gene in this cell line has been reported previously (17). B16-F10 melanoma cells continuously express luciferase (B16-F10-luc-G5; Xenogen Corp., Alameda, CA) and were maintained in DMEM with 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2.

Atelocollagen or liposome-mediated siRNA transfer

The siRNA/Atelocollagen (0.008%) complexes were prefixed to a 24-well plate (0.1–1.4 μg siRNA/50 μl/well) according to the method described previously (14). The cultured cells were plated into the complex-prefixed 24-well plate at 3.5×10^4 cells/well and the effects of siRNA transfer were then observed. The cationic liposome-mediated transfer of siRNA was performed as described by the manufacturer.

Inhibition of cell growth

For monitoring the inhibition of cell growth, the TetraColor One cell proliferation assay reagent (Seikagaku Co., Tokyo, Japan) was used according to the recommended method. The color reaction was assessed by measuring the absorbance at 450 nm with an UVmicroplate reader.

Biochemical analysis

Protein levels of human HST-1/FGF-4 in the culture supernatant and tumors were determined by using enzyme-linked immunosorbent assay (ELISA) using anti-human FGF-4 monoclonal antibody (R&D Systems, Minneapolis, MN). Absorbance was measured at a wavelength of 492 nm with a kinetic microplate reader (model 3550; Biorad, Richmond, CA).

Luciferase assays

For luciferase-based reporter gene assays, 24 μg pGL3 control vector (Promega, Madison, WI) was introduced into HEK 293 cells at 90% confluency in 10 cm dishes using LipofectAMINE™ 2000 reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. After transfection for 4 h, the cells were collected by trypsinization and plated in the 24-well dishes for siRNA transfection. Atelocollagen-mediated or conventional transfection of siRNAs into 293 cells was performed as detailed above. Cells were lysed (n = 4) on day 2 and analyzed for luciferase activity (Bright™-Glo Luciferase Assay System; Promega). Inhibition of luciferase production was normalized to the level of vehicle-treated cells. GL2 siRNA was used as control.

Analysis of siRNA delivery using *in vivo* imaging

B16-F10-luc-G5 cells were subcutaneously injected (1×10^5 cells per site) into athymic nude mice. Two days later, luciferase GL3 siRNA alone, siRNA mixed with liposome, siRNA complexed with Atelocollagen and Atelocollagen alone were injected into the tumors. For preparing the siRNA/Atelocollagen complex, an equal volume of Atelocollagen (1.0% in PBS at pH 7.4) and siRNA solution was combined and mixed by rotating for 20 min at 4°C. The siRNAs and their complexes were directly injected into the tumor (2.5 μg siRNA/50 μl/50 mm^3 tumor). The final concentration of Atelocollagen was 0.5%. The siRNA concentration used in the liposome experiments was 2.5 μg/tumor equivalent to that used in the Atelocollagen experiments. Each group contains four animals. *In vivo* bioimaging was conducted on a cryogenically cooled IVIS system (Xenogen Corp.) using LivingImage acquisition and analysis software (18). Tumor growth was not affected by these treatments. As a control for GL3 siRNA, GL2 siRNA was used.

Reporter gene labeling of tumor cells

NEC8 cells were transfected with a complex of 2 μg pEGF-PLuc plasmid DNA (Clontech, Palo Alto, CA) and 30 μl lipofection reagent (LipofectAMINE™ 2000; Invitrogen). Stable transfectants were selected in geneticin (400 μg/ml; Invitrogen) and bioluminescence was used to screen transfected clones for luciferase gene expression using the IVIS system. Clones expressing the luciferase gene were named NEC8-luc.

In vivo imaging study for orthotopic xenografts model

A total of 1.0×10^6 NEC8-luc cells were injected into mice intratesticularly. Cells were suspended in 50 μl of a serum-free medium and injected using a 26-gauge needle into both testes of 8-week-old athymic nude mice obtained from CLEA Japan (Shizuoka, Japan). Ten days after the injection of cells, tumor cell-bearing nude mice were randomly divided into four treatment groups (FGF-4 siRNA alone, FGF-4 siRNA complexed with Atelocollagen, control siRNA complexed with Atelocollagen and Atelocollagen alone). Each group consisted of four animals. The siRNAs and their complexes were injected directly into the testes (2.5 μg siRNA/50 μl/testis). The final

concentration of Atelocollagen was 0.5%. Tumor growth was monitored by measuring light emission from individual mice 21 days after siRNA administration. Three days after siRNA administration, tumors were harvested and subjected to ELISA analysis for the detection of FGF-4 protein. Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

Statistical analysis

The results are given as means ± SE. Statistical analysis was conducted using the analysis of variance with the Bonferroni correction for multiple comparisons. A P-value of 0.05 or less was considered to indicate a significant difference.

RESULTS

Atelocollagen-based delivery of siRNA into cells

To develop a method for more efficient siRNA delivery into cells, we have developed a new method for condensing and delivering siRNA using Atelocollagen. Atelocollagen, which is positively charged interacts with the negatively charged siRNA duplex to form an siRNA/Atelocollagen complex (Figure 1),

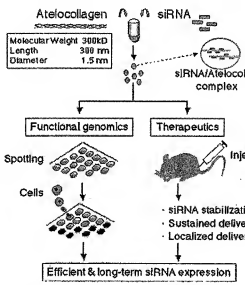

Figure 1. Schematic representation of Atelcollagen-mediated transfer of siRNA duplex for functional genomics and therapeutics. Atelocollagen is a decomposition product of type I collagen derived from the dermis of cattle with a molecular weight of 300 kDa. It is a rod-like molecule with a length and diameter of 300 and 1.5 nm, respectively. Atelocollagen, which is positively charged interacts with the negatively charged siRNA duplex to form an siRNA/Atelocollagen complex, a nanosize particle with a diameter of 100–300 nm. The siRNA/Atelocollagen complex spotted onto the well of a microplate was stable for a long period and allowed the cells to transduce and express siRNAs. The present method using Atelocollagen-based siRNA transfer is also applicable to *in vivo* siRNA transfer, since the siRNA/Atelocollagen complex is stable *in vivo*. Atelocollagen is soluble at a lower temperature but solidifies to refibrillation at a temparature over 30°C. Therefore, the siRNA/Atelocollagen complexes can be injected locally for tissue-targeting siRNA delivery. Once introduced into animals, the complex becomes a solid state and the siRNA is controlled-released for a defined period due to the biodegradable nature of Atelocollagen.

a nanosize particle with a diameter of 100–300 nm. In this system, the siRNA/Atelocollagen complexes are pre-coated on a micro-well plate on which the cells are then seeded (16) (Figure 1). Using this method, cells take up the siRNA/Atelocollagen complex and siRNA exerts a gene silencing effect. To examine whether Atelocollagen blocks degradation of siRNA from nuclease, naked siRNA, siRNA/liposome complex and siRNA/Atelocollagen complex were incubated in the presence of RNase (0.1 μg/μl) for 0, 5, 15, 30, 45 and 60 min at 37°C followed by agarose gel electrophoresis. The results indicated that the siRNA/Atelocollagen complex showed partial resistance to degradation of siRNA in the presence of nuclease (Figure 2). In addition, ~50% of the siRNA were incorporated into the Atelocollagen, which suggests non-incorporated siRNAs are degraded (data not shown). Furthermore, Atelocollagen demonstrated 40–60% efficiency of cellular uptake of FITC-labeled siRNAs 24 h after transfection (data not shown). To evaluate the efficiency of Atelocollagen-mediated transfer technology using well-characterized siRNA, we employed a luciferase reporter

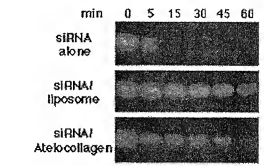

Figure 2. Atelocollagen blocks degradation of siRNA by RNase A. Naked siRNA, siRNA/liposome and siRNA/Atelocollagen complexes were incubated in the presence of RNase A for 0, 5, 15, 30, 45 and 60 min at 37°C and then agarose gel electrophoresed. The presence of siRNA was revealed by ethidium bromide staining.

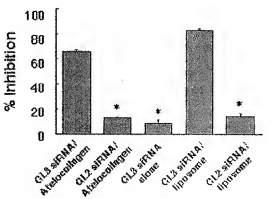

Figure 3. Characteristics of Atelocollagen-mediated siRNA transfer technology. Inhibitory effect of luciferase production in 293 cells. The GL3 siRNA duplexes were transfected into pGL3 control plasmid transfected 293 cells by polycation-reagent or complexed with Atelocollagen. Luciferase activity was measured on day 2 ($n = 4$, mean ± SE). *, $P < 0.001$ versus GL3 siRNA/Atelocollagen and GL3 siRNA liposome-treated cells. As a control for GL3 siRNA, GL2 siRNA was used.

gene system in 293 cells. As shown in Figure 3, our Atelocollagen-mediated siRNA delivery technology exhibited an inhibitory effect as efficient as that in the conventional liposome transfer method.

In the next experiment, we employed human testicular tumor cells, NEC8, which showed high levels of HST-1/FGF-4 mRNA expression (17) and specifically inhibited cell growth by suppression of HST-1/FGF-4 (19). An Atelocollagen-mediated delivery of human HST-1/FGF-4 siRNA was performed to inhibit NEC8 cell growth. The inhibitory effect of HST-1/FGF-4 siRNA was dose-dependent and 1.4 μg per 3.5×10^4 cells produced maximum inhibition (Figure 4A). At a dose of 1.4 μg per 3.5×10^4 cells showed ~10% toxicity by the trypan blue exclusion. Therefore, we used human HST-1/FGF-4 siRNA at a subnaximal dose of 0.7 μg per 3.5×10^4 NEC8 cells for further studies. The NEC8 cells transfected with siRNA plus polycation reagent showed an inhibitory effect for maximum of 4 days post-transfection and there was no inhibition of cell growth thereafter (Figure 4B). In addition, siRNA alone and liposome alone showed no significant inhibitory effect (data not shown). In contrast, HST-1/FGF-4 siRNA complexed with Atelocollagen displayed inhibition of cell growth for at least 7 days in culture. To verify further that cell growth inhibition reflected a gene-specific silencing event, HST-1/FGF-4 protein production in NEC8 cells was investigated by ELISA (20). As shown in Figure 5, HST-1/FGF-4 protein levels were significantly inhibited when cells were transfected with the siRNA/Atelocollagen complex. Taken together, these data show that the Atelocollagen stabilized siRNA and thereby siRNA/Atelocollagen complex was able to produce an efficient and a long-term gene silencing effect *in vitro*.

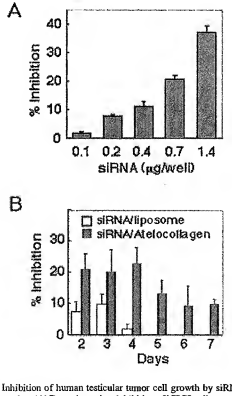

Figure 4. Inhibition of human testicular tumor cell growth by siRNA/Atelocollagen complex. (A) Dose-dependent inhibition of NEC8 cell growth. Human HST-1/FGF-4 siRNAs (0.1–1.4 μg) complexed with 0.008% Atelocollagen were transduced into NEC8 cells. Cell proliferation was measured at 2 days after treatment ($n = 4$, mean ± SE). (B) Long-term inhibition of NEC8 cell growth by siRNA/Atelocollagen complex. HST-1/FGF-4 siRNA (0.7 μg) was transfected into NEC8 cells by polycation-reagent and complexed with 0.008% Atelocollagen ($n = 6$, mean ± SE).

Enhanced gene silencing by siRNA/Atelocollagen complex *in vivo*

To test whether Atelocollagen-mediated siRNA transfer is valid for gene silencing *in vivo* (Figure 1), animal experiments were performed on mice bearing a luciferase-producing melanoma. Non-invasive *in vivo* bioluminescence imaging analysis showed that luciferase expressions in the tumor of mice injected with GL3 siRNA alone and liposome-complexed siRNA were maximally inhibited at 2–3 days after injection, and increased thereafter. In contrast, mice administered with the siRNA/Atelocollagen complex showed a relatively strong and sustained inhibition of luciferase expression *in vivo* (Figures 6A and B). As previously shown, radiolabeled siRNA mixed with Atelocollagen existed in the tumors for at least a week and remained intact (21). These results suggest that an Atelocollagen-mediated *in vivo* transfer of siRNA could be a powerful and simple method to study loss-of-function of genes in animals.

Inhibition of tumor growth by siRNA/Atelocollagen complex

Testicular injections of NEC8 cell lines in Balb/c nude mice demonstrated relevant tumor biology (19). In this study, the NEC8 cell line was labeled through expression of a stable integrant of the luciferase gene. Athymic nude mice laden

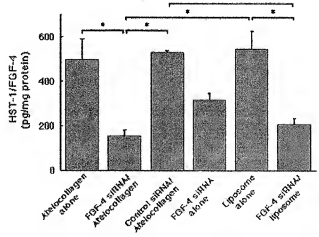

Figure 5. Silencing effect on HST-1/FGF-4 protein production in NEC8 cells. HST-1/FGF-4 siRNA (0.7 μg) complexed with 0.008% Atelocollagen was transduced into NEC8 cells. As a control, an Atelocollagen complex with non-specific control siRNA duplex that shows no silencing effect on human HST-1/FGF-4 was used (control-siRNA/Atelocollagen). Production of HST-1/FGF-4 protein was measured by ELISA 3 days after the transfer of siRNA ($n = 3$, mean ± SE). *, $P < 0.05$.

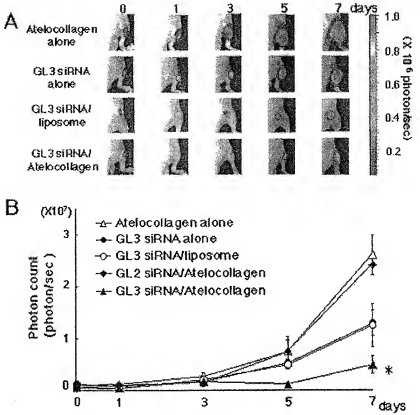

Figure 6. *In vivo* imaging of gene silencing effect of siRNA/Atelocollagen complex. (A) Luciferase GL3 siRNA (2.5 μg) complexed with 0.5% Atelocollagen was administered into mice and luciferase expression of xenografted tumors was monitored by *in vivo* imaging analysis. As a control, mice administered with siRNA alone, siRNA complexed with liposome and Atelocollagen alone were investigated. Color bar represents signal intensity code over body surface area. (B) Luciferase gene expression was measured periodically and is represented as photon/s. Number of tumors at each time point is 4. As a control for GL3 siRNA, GL2 siRNA was used. Data represent the mean ± SE. *, $P < 0.05$ versus Atelocollagen treatment.

with a testicular injection of NEC8-luc cells were randomly selected for treatment with HST-1/FGF-4 siRNA alone, siRNA complexed with Atelocollagen or Atelocollagen alone. Previously, bioluminescence imaging of orthotopic xenografts in mice demonstrated a linear correlation between tumor bioluminescence and tumor volume (18,22). Tumor growth was inhibited by treatment with human HST-1/FGF-4 siRNA complexed with Atelocollagen. At 21 days following treatment, tumor volume in mice treated with siRNA complexed with Atelocollagen was smaller than that in the control mice treated with Atelocollagen alone (Figure 7A and B). In contrast, tumors treated with siRNA alone and control siRNA/Atelocollagen showed no significant volume reduction. Furthermore, the FGF-4 siRNA/Atelocollagen complex significantly inhibited the production of FGF-4 in the tumors (Figure 7C) and this inhibition lasted for 20 days. Therefore, the Atelocollagen-mediated siRNA transfer is a significant novel method for inhibition of tumor growth *in vivo*.

DISCUSSION

Silencing of gene expression by siRNAs is rapidly becoming a powerful tool for the genetic analysis of a wide variety of mammalian cells. Although in the original studies, the expression of siRNA in mammalian cells was achieved via the transfection of double-stranded oligonucleotides, subsequent studies described the limited duration of the gene silencing effect. To overcome this problem, the use of plasmids to achieve a long-term and stable expression of siRNA was established (23–25). In addition, several groups have described the use of adenoviral vectors (9), retroviral vectors (26) and self-inactivating lentiviral vectors (27) for siRNA delivery. However, viral vectors suffer from the problem of severe side effects. Although the 'hydrodynamic transfection method' and a liposome transfection method were recently reported for siRNA delivery into animals (8,28), none is suitable for clinical use. Therefore, the development of safe non-vector-based siRNA delivery systems is critical for the future of siRNA-based therapies. Here, we used an Atelocollagen-mediated siRNA transfer in an *in vitro* and *in vivo* germ cell tumor-suppression model. Because Atelocollagen allowed increased cellular uptake, nuclease resistance and prolonged release of siRNAs, Atelocollagen complexed with siRNA rather than siRNA alone or a polycation transfer method resulted in stronger gene silencing effects over other methods. It is known that Atelocollagen has the ability to transfer genes to both dividing and non-dividing

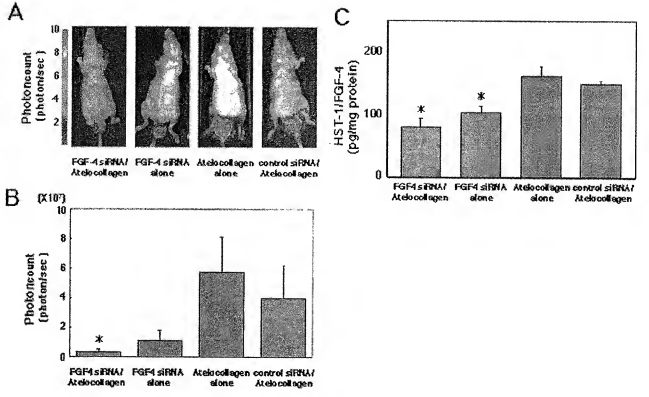

Figure 7. Effect of siRNA/Atelocollagen complex on the growth of a xenograft tumor. (A) Human HST-1/FGF-4 siRNA (2.5 μg) complexed with 0.5% Atelocollagen was transduced into an orthotopic germ cell tumor of NEC8 cells expressing the luciferase gene. Representative images at 21 days after treatment are shown. As a control, an Atelocollagen complex with non-specific control siRNA duplex that shows no silencing effect on human HST-1/FGF-4 was used (control siRNA/Atelocollagen). (B) Measurements of a xenograft tumor bioluminescence at 21 days after treatment. Data represent the mean ± SE. *, $P < 0.05$ versus Atelocollagen alone treatment. (C) Evaluation of HST-1/FGF-4 protein expression in tumor tissue extracts 3 days after treatment. Protein levels were quantified by ELISA. Data represent the mean ($n = 4$) ± SE. *, $P < 0.05$ versus Atelocollagen alone and control siRNA/Atelocollagen treatment.

cells. Thus, for clinical applications in RNAi therapy, an Atelocollagen-based siRNA transfer system represents an attractive method to achieve maximal function of siRNA-based gene silencing *in vivo*.

One technical problem associated with siRNA transfer *in vivo* is the targeting of siRNA delivery to a specific tissue. For this purpose, our Atelocollagen-based transfer method has great potential for site-specific transportation of target siRNAs because the complex of siRNA/Atelocollagen becomes solid when transplanted and remains so for a defined period *in vivo*. In addition, an Atelocollagen complex can be delivered as micro-particles for intravenous injection, making systemic delivery of siRNA possible. A recent report showed the potential for Atelocollagen-mediated systemic antisense therapeutics for inflammatory disease (29). Following *in vivo* administration, the incorporated siRNAs are slowly released over an extended period of time. This eliminates the need for multiple injections of siRNA and siRNA vectors, in lessened side effects.

Although siRNAs are thought to be too short to induce interferon expression, recent reports has shown that siRNA sequences and their method of delivery may trigger an interferon response (30,31). Therefore, alternative strategies are needed to reduce the induction of non-specific side effects. In this regard, our Atelocollagen-mediated non-vector transfer method is an attractive strategy to deliver siRNAs *in vivo*, since our Atelocollagen has low-toxicity and is low-immunogenic, and hence unlikely to stimulate interferon expression *in vivo*.

Finally, based on the ability of Atelocollagen to achieve the sustained release of siRNA and to enhance the stability of siRNA *in vivo*, our novel delivery method demonstrates potential for use as a therapeutic tool for the delivery of siRNA.

ACKNOWLEDGEMENTS

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EXHIBIT 15

Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs

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RNA interference (RNAi) holds considerable promise as a therapeutic approach to silence disease-causing genes, particularly those that encode so-called 'non-druggable' targets that are not amenable to conventional therapeutics such as small molecules, proteins, or monoclonal antibodies. The main obstacle to achieving *in vivo* gene silencing by RNAi technologies is delivery. Here we show that chemically modified short interfering RNAs (siRNAs) can silence an endogenous gene encoding apolipoprotein B (apoB) after intravenous injection in mice. Administration of chemically modified siRNAs resulted in silencing of the apoB messenger RNA in liver and jejunum, decreased plasma levels of apoB protein, and reduced total cholesterol. We also show that these siRNAs can silence human apoB in a transgenic mouse model. In our *in vivo* study, the mechanism of action for the siRNAs was proven to occur through RNAi-mediated mRNA degradation, and we determined that cleavage of the apoB mRNA occurred specifically at the predicted site. These findings demonstrate the therapeutic potential of siRNAs for the treatment of disease.

RNAi has been applied widely as a target validation tool in post-genomic research, and it represents a potential strategy for *in vivo* target validation and therapeutic product development[1]. *In vivo* gene silencing with RNAi has been reported using both viral vector delivery[2] and high-pressure, high-volume intravenous (i.v.) injection of synthetic siRNAs[3], but these approaches have limited if any clinical use. *In vivo* gene silencing has also been reported after local, direct administration (intravitreal, intranasal and intrathecal) of siRNAs to sequestered anatomical sites in models of choroidal neovascularization[4], lung ischaemia-reperfusion injury[5] and neuropathic pain[6], respectively. These reported approaches demonstrate the potential for delivery to organs such as the eye, lungs and central nervous system. However, there are no published reports of systemic activity for siRNAs towards endogenous targets after conventional and clinically acceptable routes of administration. A critical requirement for achieving systemic RNAi *in vivo* is the introduction of 'drug-like' properties, such as stability, cellular delivery and tissue bioavailability, into synthetic siRNAs.

Conferring drug-like properties on siRNAs

In exploring the potential of synthetic siRNAs to silence endogenous target genes, we found that chemically stabilized and cholesterol-conjugated siRNAs[7] have markedly improved pharmacological properties *in vitro* and *in vivo*. Chemically stabilized siRNAs with partial phosphorothioate backbone and 2′-*O*-methyl sugar modifications on the sense and antisense strands showed significantly enhanced resistance towards degradation by exo- and endonucleases in serum and in tissue homogenates. The conjugation of cholesterol to the 3′ end of the sense strand of a siRNA molecule by means of a pyrrolidine linker (thereby generating chol-siRNA) did not result in a significant loss of gene-silencing activity in cell culture. Furthermore, unlike unconjugated siRNAs, a chol-siRNA directed to luciferase (chol-luc-siRNA) showed reduction in luciferase activity in HeLa cells transiently expressing luciferase, with a half-maximal inhibitory concentration (IC_{50}) of about 200 nM in the absence of transfection reagents or electroporation.

Binding of chol-siRNAs to human serum albumin (HSA) was determined by surface plasmon resonance measurement (data not shown). Unconjugated siRNAs demonstrated no measurable binding to HSA, whereas chol-siRNAs bound to HSA with an estimated dissociation constant (K_d) of 1 μM. Presumably because of enhanced binding to serum proteins, chol-siRNAs administered to rats by i.v. injection showed improved *in vivo* pharmacokinetic properties as compared to unconjugated siRNAs. After i.v. injection in rats at 50 mg kg^{-1}, radioactively labelled chol-siRNAs had an elimination half life (two compartments), $t_{1/2}$ of 95 min and a corresponding plasma clearance (C_L) of 0.5 ml min^{-1}, whereas unconjugated siRNAs had a $t_{1/2}$ of 6 min and C_L of 17.6 ml min^{-1}. As measured by an RNase protection assay (RPA), chol-siRNAs showed broad tissue biodistribution 24 h after injection in mice. Although no detectable amounts of unconjugated siRNAs were observed in tissue samples, significant levels of chol-siRNAs were detected in liver, heart, kidney, adipose, and lung tissue samples. Together, these studies demonstrate that cholesterol conjugation significantly improves *in vivo* pharmacological properties of siRNAs.

Selection of apoB as an endogenous gene target

Apolipoprotein B is the essential protein for formation of low-density lipoproteins (LDL) in metabolism of dietary and endogenous cholesterol, and is the ligand for the LDL receptor[8]. Mouse apoB is a large protein of 4,515 amino acids and is expressed predominantly in liver and jejunum. apoB mRNA is subject to post-transcriptional editing, and the unedited and edited transcripts encode the full-length protein apoB-100, and a carboxy-terminal truncated isoform, apoB-48, respectively. In mice, editing of apoB mRNA occurs in both the liver and jejunum: apoB-48 is the predominant protein form in the jejunum and both apoB-48 and apoB-100 are expressed in the liver. Heterozygous knockout mice for apoB show a 20% decrease in cholesterol levels and are resistant to

diet-induced hypercholesterolaemia[9].

Serum levels of apoB, LDL and cholesterol correlate significantly with increased risk of coronary artery disease (CAD). A diminished number of functional LDL receptors on the cell surface, disrupting receptor-mediated removal of apoB-containing LDL from circulation, has been identified as the basis for familial hypercholesterolaemia (FH)[10]. Patients with homozygous and heterozygous FH have accelerated CAD leading to premature atherosclerosis and cardiac mortality. Conversely, patients with hypobetalipoproteinaemia have reduced levels of LDL and cholesterol and are at reduced risk for CAD[11]. Accordingly, lowering of serum cholesterol and LDL levels is a predominant clinical strategy for management of CAD and is achieved by modification of dietary sources of cholesterol and/or inhibition of endogenous cholesterol synthesis with pharmacological therapies. Notwithstanding significant improvements in the management of CAD with these approaches, millions of patients remain at significant risk for CAD and its clinical sequelae—acute coronary syndromes such as myocardial infarction and cardiac mortality—due to advanced atherosclerosis from intractably high levels of cholesterol and LDL. Clearly, new therapeutic strategies are needed. Accordingly, apoB, a protein not amenable to inhibition by conventional small-molecule- or protein-based therapeutics, was selected as a potential clinical target for development of siRNA therapeutics.

Using conventional bioinformatics, 84 siRNAs specific for both human and mouse apoB mRNA were designed and synthesized (data not shown). These apoB-siRNAs were screened for their ability to reduce apoB mRNA and protein levels, as measured by polymerase chain reaction with reverse transcription (RT–PCR) and enzyme-linked immunosorbent assay (ELISA), respectively, in HepG2 liver cells after transfection at a concentration of 100 nM. Five apoB-siRNAs were identified that reduced both mRNA and protein levels by >70%. Because exonucleolytic degradation is the predominant mechanism for siRNA degradation in serum, two selected apoB-siRNAs (apoB-1-siRNA and apoB-2-siRNA) and one four-nucleotide mismatch control for apoB-1-siRNA (mismatch-siRNA) were stabilized at the 3′ end of the sense and antisense strands by phosphorothioate backbone modifications and additional incorporation of two 2′-*O*-methyl nucleotides at the 3′ end of the antisense strand. Chol-siRNAs were synthesized by linkage of cholesterol to the 3′ end of the sense strand via a pyrrolidine linker. Chol-apoB-1-siRNA was significantly more stable than unconjugated apoB-1-siRNA in human serum: gel electrophoresis showed >50% intact chol-apoB-1-siRNA after a 1 h incubation at 37 °C compared with <5% intact unconjugated apoB-1-siRNA. Similar data were obtained for chol-apoB-2-siRNA, although this siRNA was less stable than chol-apoB-1-siRNA. Dose response curves for the activity of conjugated and unconjugated apoB-specific and control siRNAs were measured in HepG2 cells using transfection. Two conjugated control siRNAs (chol-luc-siRNA and chol-mismatch-siRNA) showed no significant inhibition of apoB protein expression at concentrations as high as 30 nM. In contrast, three specific siRNAs (unconjugated apoB-1-siRNA, chol-apoB-1-siRNA and chol-apoB-2-siRNA) showed dose-dependent silencing of apoB protein expression based on apoB ELISA measurements—IC_{50} values of 0.5 nM, 5 nM and 8 nM were calculated, respectively.

In vivo studies with modified siRNAs

To demonstrate the ability of chol-apoB-siRNAs to silence apoB expression *in vivo*, experiments were first performed in C57BL/6

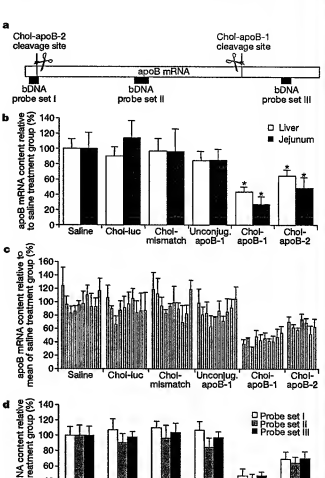

Figure 2 *In vivo* silencing of murine apoB mRNA by siRNAs in wild-type mice. Treatment groups comprised saline control ($n = 10$), chol-luc-siRNA control ($n = 10$), chol-mismatch-siRNA control ($n = 10$), unconjugated apoB-1-siRNA ($n = 10$), chol-apoB-1-siRNA ($n = 10$) and chol-apoB-2-siRNA ($n = 7$). bDNA measurements were performed with probe set II. Error bars represent the standard deviation (s.d.) of the mean. Statistical analysis was by analysis of variance (ANOVA) with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline control animals. a, Schematic representation of the apoB mRNA illustrating the binding regions of three bDNA probe sets in relation to the two siRNA cleavage sites. b, Effects of siRNA administration on mean apoB mRNA levels. c, apoB mRNA levels from individual mice treated with saline or siRNAs. Data are mean values from three liver samples from each individual animal. d, Effects of siRNA administration on the reduction of apoB mRNA measured by bDNA assays using three different probe sets.

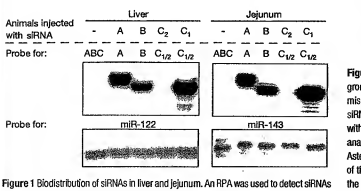

Figure 1 Biodistribution of siRNAs in liver and jejunum. An RPA was used to detect siRNAs in pooled liver and jejunum tissue lysates from animals injected with saline (–), chol-luc-siRNA (A), chol-mismatch-siRNA (B), unconjugated apoB-1-siRNA (C_2) or chol-apoB-1-siRNA (C_1). Detection by RPA of endogenous miRNAs in liver (miR-122) and jejunum (miR-143) served as an internal loading control.

mice fed a normal chow diet. siRNAs were administered by tail-vein injection with normal volume (0.2 ml) and normal pressure. Biodistribution of siRNAs was assessed by RPA of siRNAs in tissue samples from liver and jejunum obtained 24 h after the last injection. Significant levels of chol-luc-siRNA, chol-apoB-1-siRNA and chol-mismatch-siRNA were detected in liver and jejunum (100–200 ng g^{-1} tissue for chol-apoB-1-siRNA), whereas levels of unconjugated apoB-1-siRNA were below our detection limit (Fig. 1). Levels of chol-apoB-2-siRNA were also detected but at levels approximately 10% of those observed for other chol-siRNAs.

The primary measure of RNAi-mediated effects is the reduction (that is, silencing) of the target mRNA. To measure silencing of apoB mRNA, we used a branched-DNA (bDNA) detection method and bDNA probes (Fig. 2a) to quantify apoB mRNA levels in liver and jejunum, two organs where apoB is known to be expressed. As shown in Fig. 2b, mice treated with chol-apoB-1-siRNA and chol-apoB-2-siRNA showed statistically significant reductions (mean ± s.d.; 57 ± 6% and 36 ± 8%, respectively) in apoB mRNA levels in liver samples as compared with saline control ($P < 0.0001$). In jejunum tissue samples, mice injected with chol-apoB-1-siRNA and chol-apoB-2-siRNA showed an even more substantial reduction in apoB mRNA levels of 73 ± 10% and 51 ± 13%, respectively, as compared with saline control ($P < 0.0001$). Individual animal results for apoB mRNA reduction in the liver are shown in Fig. 2c and demonstrate the consistent and robust effect observed for specific chol-siRNAs as compared with other treatment groups. Similar results were observed for apoB mRNA reduction in the jejunum from individual animals (data not shown). Owing to the extended length of the apoB mRNA, two additional probes at the distal ends of the apoB open reading frame (ORF) were designed. As measured with the three divergent probe sets, identical levels of apoB mRNA reduction were detected for animals treated with chol-apoB-1-siRNA and chol-apoB-2-siRNA (Fig. 2d). These data suggest a uniform and rapid degradation of apoB mRNA after treatment with chol-apoB-siRNAs, and argue against the potential existence of truncated amino-terminal apoB protein fragments translated from incompletely degraded siRNA-cleavage products, as has been reported for ribozyme-mediated cleavage of apoB mRNA[12].

Silencing of the apoB mRNA would be expected to result in a corresponding reduction in apoB protein levels. An ELISA-based method specific for detection of apoB-100 protein was used to measure the effects of chol-apoB-siRNA treatment on plasma levels of apoB protein. In addition to the effects on apoB mRNA levels, treatment with chol-apoB-1-siRNA and chol-apoB-2-siRNA reduced plasma levels of apoB-100 protein 24 h after siRNA treatment by 68 ± 14% and 31 ± 18%, respectively, compared with levels in saline-treated control animals (Fig. 3). These results achieved statistical significance ($P < 0.0001$) for the group treated with the more potent and stable chol-apoB-1-siRNA. As the LF3 antibody used in this study recognizes only apoB-100, and not apoB-48, the observed apoB-100 reduction may underestimate the full effect of chol-apoB-1-siRNA at the protein level.

To confirm the physiological relevance of apoB mRNA silencing on lipoprotein metabolism, we characterized the effect of siRNA treatment and the resulting reduction of apoB protein levels on lipoprotein profiles and cholesterol levels. Using an NMR-based method, complete lipoprotein profiles were generated and concentrations of chylomicrons, very-low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL) particles were calculated (Fig. 4a). As expected, HDL represented the predominant lipoprotein fraction in mouse plasma. Similar to results observed in heterozygous knockout mice for apoB[9], treatment with chol-apoB-1-siRNA resulted in a 25% reduction in HDL particle

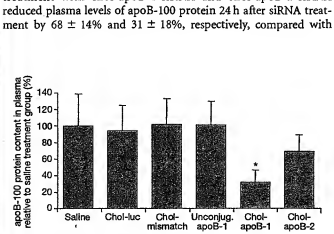

Figure 3 Effects of siRNA administration on apoB-100 protein levels. Average plasma levels of apoB-100 protein for the different treatment groups as measured by ELISA. Error bars represent the s.d. of the mean. Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline control animals.

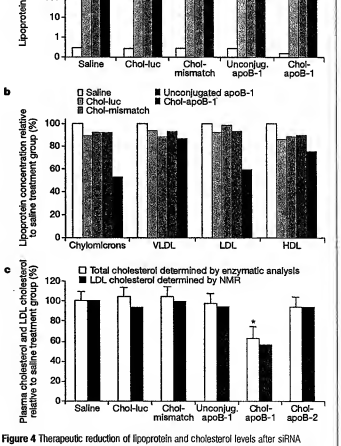

Figure 4 Therapeutic reduction of lipoprotein and cholesterol levels after siRNA treatment. a, Lipoprotein profile of pooled plasma samples from treatment groups determined by NMR analysis. b, Relative reduction of lipoprotein classes for the siRNA treatment groups normalized against the average levels of saline control group. c, Effects of siRNA administration on plasma cholesterol and LDL cholesterol. Plasma cholesterol was determined by enzymatic assay and LDL cholesterol calculated from NMR measurements. Error bars represent the s.d. of the mean. Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline and chol-mismatch-siRNA control animals. NMR data are based on single measurements of pooled plasma from treatment groups.

concentration (Fig. 4b). Furthermore, treatment of mice with chol-apoB-1 siRNA resulted in an almost 50% reduction of chylomicron levels and an approximately 40% reduction in LDL levels, whereas VLDL levels were not altered. Treatment with either of the control siRNAs did not change the lipoprotein profile significantly. In addition to reductions in lipoprotein concentrations, *in vivo* silencing of apoB by chol-apoB-1-siRNA led to a significant reduction ($37 \pm 11\%$; $P < 0.0001$) of total plasma cholesterol as compared with saline control animals (Fig. 4c). Treatment with the less potent chol-apoB-2-siRNA failed to show significant reductions in cholesterol, consistent with the reduced activity of this chol-siRNA on apoB mRNA and protein levels. Treatment with chol-apoB-1-siRNA also resulted in a 44% decrease in LDL-associated cholesterol, consistent with the effects observed on apoB protein levels. In aggregate, the effects on cholesterol reduction and lipoprotein profiles would be considered highly clinically significant in patients with hypercholesterolaemia, and actually exceed the level of cholesterol reduction observed in heterozygous apoB knockout mice[9].

To extend our findings of *in vivo* silencing by chol-apoB-siRNAs in normal mice, we performed an additional study in a human apoB transgenic mouse model[13]. These mice express human apoB-100 in liver and have elevated levels of apoB as compared with normal mice; when fed a high-fat diet, these mice develop severe atherosclerosis[14]. In our experiments, we administered saline, chol-mismatch-siRNA and chol-apoB-1-siRNA to apoB transgenic mice fed a normal chow diet. As shown in Fig. 5, chol-apoB-1-siRNA brought about a significant reduction of endogenous murine apoB expressed in both liver and jejunum tissue samples ($P < 0.0001$, relative to saline and chol-mismatch-siRNA treatment). Relative to the saline control, levels of murine apoB mRNA were reduced by $57 \pm 10\%$ in liver and $42 \pm 12\%$ in jejunum. In addition, chol-apoB-1-siRNA, which was selected in part owing to its sequence identity to both human and mouse apoB, showed significant silencing of the human transgene expressed in the liver, where human apoB mRNA was silenced by $60 \pm 10\%$ ($P < 0.0001$). In contrast to these effects, chol-mismatch-siRNA showed no effect on mouse or human apoB mRNA levels. These results confirm the effect of specific chol-siRNAs on apoB silencing in a different mouse model. Moreover, this specific chol-siRNA was shown to silence a transgenic human mRNA *in vivo*.

An important consideration for siRNA-mediated inhibition of gene expression is whether the observed effects are specific and not due to nonspecific "off target" effects[15] and potential interferon responses[16], which have been reported with siRNAs *in vitro* and other oligonucleotide-based approaches *in vivo*. In our experiments, the effects of apoB-specific, cholesterol-conjugated siRNAs were seen with two divergent siRNAs targeting separate sequence regions of the apoB mRNA. Furthermore, the *in vivo* silencing of apoB by these siRNAs was specific as neither an irrelevant siRNA (chol-luc-siRNA) nor a mismatch control siRNA (chol-mismatch-siRNA)—although present at comparable concentrations in liver and jejunum—mediated a significant reduction in apoB mRNA, plasma apoB protein levels, or total cholesterol. Finally, the silencing of apoB mRNA by chol-apoB-siRNAs in liver as measured by bDNA assay and normalization to GAPDH mRNA was also demonstrated with normalization to three other liver mRNAs, including factor VII, glucose-6-phosphatase and VEGF (Supplementary Fig. 1).

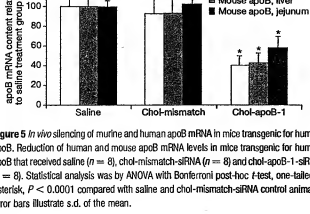

Figure 5 *In vivo* silencing of murine and human apoB mRNA in mice transgenic for human apoB. Reduction of human and mouse apoB mRNA levels in mice transgenic for human apoB that received saline ($n = 8$), chol-mismatch-siRNA ($n = 8$) and chol-apoB-1-siRNA ($n = 8$). Statistical analysis was by ANOVA with Bonferroni post-hoc *t*-test, one-tailed. Asterisk, $P < 0.0001$ compared with saline and chol-mismatch-siRNA control animals. Error bars illustrate s.d. of the mean.

Determination of *in vivo* mechanism of action

To prove that the *in vivo* activity was due to siRNA-directed cleavage, we characterized specific mRNA cleavage products using a modified 5′-RACE (rapid amplification of cDNA ends) technique previously used to demonstrate microRNA (miRNA)-directed mRNA cleavage in plants[17] and mouse embryos[18]. As it relates to the specific cleavage of apoB mRNA by apoB-1-siRNAs, total RNA from mice in the different treatment groups was isolated, and then PCR was used to reveal fragments of the predicted length in animals receiving chol-apoB-1-siRNA treatment (Fig. 6a). Identity of the PCR products was confirmed by direct sequencing of the excised bands, which demonstrated that cleavage occurred at the predicted position for the siRNA duplex. Indeed, sequencing revealed cleavage after position 10,061 of the apoB ORF, exactly ten nucleotides downstream of the 5′ end of the siRNA antisense strand. Specific cleavage fragments were detected in both liver and jejunum of animals receiving chol-apoB-1-siRNA treatment (Fig. 6b). No fragments were detected in tissues of animals receiving control siRNAs (chol-luc-siRNA or chol-mismatch-siRNA) or saline. As expected, in this 5′-RACE experiment of apoB mRNA cleavage mediated by chol-apoB-1-siRNA, no fragments were detected in tissues from animals receiving the alternative apoB-specific siRNA (chol-apoB-2-siRNA). Notably, a low level of specific cleavage product was detected in the jejunum of animals receiving the unconjugated apoB-1-siRNA despite no evidence for significant knockdown of total apoB mRNA levels by this siRNA. This indicates

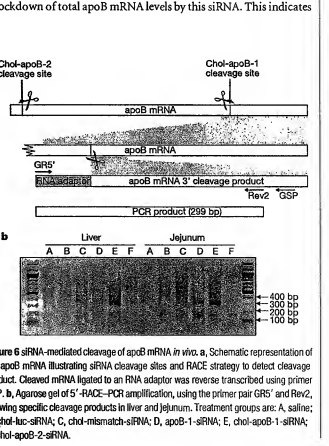

Figure 6 siRNA-mediated cleavage of apoB mRNA *in vivo*. **a**, Schematic representation of the apoB mRNA illustrating siRNA cleavage sites and RACE strategy to detect cleavage product. Cleaved mRNA ligated to an RNA adaptor was reverse transcribed using primer GSP. **b**, Agarose gel of 5′-RACE–PCR amplification, using the primer pair GRS′ and Rev2, showing specific cleavage products in liver and jejunum. Treatment groups are: A, saline; B, chol-luc-siRNA; C, chol-mismatch-siRNA; D, apoB-1-siRNA; E, chol-apoB-1-siRNA; F, chol-apoB-2-siRNA.

that some unconjugated apoB-1-siRNA is able to enter epithelial cells of the jejunum after systemic administration despite lacking cholesterol conjugation. Therefore, these data demonstrate that inhibition of apoB was achieved by an RNAi mechanism of action. To our knowledge, this is the first demonstration of silencing of an endogenous gene in mammals by a mechanism of RNAi-mediated degradation of the target mRNA.

Discussion

Our findings demonstrate that RNAi can be used to silence endogenous genes involved in the cause or pathway of human disease with a clinically acceptable formulation and route of administration by means of systemic delivery. In our study, we have shown that the mechanism of action for chemically modified siRNAs was by RNAi-mediated degradation of the target mRNA. Chol-apoB-siRNAs, but not unconjugated apoB-siRNAs, showed biological activity, demonstrating an important role for cholesterol conjugation of siRNAs to achieve systemic *in vivo* activity, and suggesting the opportunity to further optimize systemic activity through chemical conjugation strategies. Indeed, further optimization is warranted to achieve improved *in vivo* potency for chol-siRNAs at doses and dose regimens that are clinically acceptable. Nevertheless, these findings hold promise for the development of a new class of therapeutics that harnesses the RNAi mechanism. Of particular interest is the use of RNAi therapeutics to silence genes (such as the apoB gene) or mutated or variant alleles whose proteins are refractory to the discovery of traditional small molecules or biotherapeutic drugs. □

Methods

Synthesis of siRNAs

The siRNAs used in this study consisted of a 21-nucleotide sense strand and a 23-nucleotide antisense strand resulting in a two-nucleotide overhang at the 3′ end of the antisense strand. apoB-1-siRNA (ORF position 10049–10071): sense 5′-GUCAUCACAC UGAAUACCAA*U-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGAC*A*C-3′; chol-apoB-1-siRNA: sense 5′-GUCAUCACACUGAAUACCAAU*chol-3′, antisense 5′-AUUGGUAUUCAGUGUGAUGAC*A*C-3′; chol-mismatch-siRNA: sense 5′-GUGAUCAGACUCAAUACGAAU*chol-3′, antisense 5′-AUUCGUAUUGAGUCUGA UCA*C*A*C-3′; chol-apoB-2-siRNA (ORF position 327–349): sense 5′-AGGUGUAUGGC UUCAACCCUG*chol-3′, antisense 5′-CAGGGUUGAAGCCAUACACC*U*U-3′; chol-luc-siRNA: sense 5′-GAACUGUGUGUGAGAGGUCCU*chol-3′, antisense 5′-AGGAC CUCUCACACACAGUUC*g*C-3′. The lower-case letters represent 2′-*O*-methyl-modified nucleotides; asterisks represent phosphorothioate linkages.

RNA oligonucleotides were synthesized using commercially available 5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoramidite monomers of uridine (U), 4-*N*-benzoylcytidine (C^{Bz}), 6-*N*-benzoyladenosine (A^{Bz}) and 2-*N*-isobutyrylguanosine (G^{iBu}) with 2′-*O*-t-butyldimethylsilyl protected phosphoramidites and the corresponding 2′-*O*-methyl phosphoramidites according to standard solid phase oligonucleotide synthesis protocols[19]. After cleavage and de-protection, RNA oligonucleotides were purified by anion-exchange high-performance liquid chromatography and characterized by ES mass spectrometry and capillary gel electrophoresis. RNA with phosphorothioate backbone at a given position was achieved by oxidation of phosphite with Beaucage reagent[20] during oligonucleotide synthesis. Chol-siRNAs were synthesized using the same protocols as above except that the RNA synthesis started from a controlled-pore glass solid support carrying a cholesterol-aminocaproic acid-pyrrolidine linker (V.Ke., K.G.R. and M.M., unpublished data). For this support, the first nucleotide linkage was achieved using a phosphorothioate linkage to provide additional 3′-exonuclease stability. To generate siRNA from single strands, equimolar amounts of complementary sense and antisense strands were mixed and annealed, and siRNAs were further characterized by native gel electrophoresis.

In vitro activity and stability assays

To determine *in vitro* activity of siRNAs, HepG2 cells were transfected with siRNAs using oligofectamine (Invitrogen) and siRNA concentrations ranging from 0.1, 0.3, 1, 3, 10 to 30 nM. apoB protein concentration was determined from cell culture supernatant by a sandwich ELISA capturing apoB with a polyclonal goat anti-human apoB antibody (Chemicon International). apoB detection was performed with a horseradish peroxidase-conjugated goat anti-human apoB-100 polyclonal antibody (Academy Bio-Medical Company). The remaining apoB protein content was calculated as the ratio of apoB protein concentration in the supernatant of cells treated with the apoB-specific siRNA duplex to the apoB concentration in the supernatant of cells treated with an unspecific control siRNA duplex. Mouse serum (Sigma-Aldrich Chemie GmbH) was used for stability assays. Double-stranded RNAs (5 μM) were incubated in 95% serum, and the mixture was incubated at 37 °C for various lengths of time (for example, 0, 15 or 30 min, or 1, 2, 4, 8, 16 or 24 h). siRNAs were isolated by hot phenol extraction in the presence of sodium dodecyl sulphate followed by ethanol precipitation. Re-suspended RNA samples were run on a denaturing 14% polyacrylamide gel containing 20% formamide for 2 h at 45 mA. RNA bands were visualized by staining with the 'Stains-All' reagent (Sigma-Aldrich Chemie GmbH) according to the manufacturer's instructions.

In vivo silencing experiments

C57BL/6 mice received, on three consecutive days, tail vein injections of saline or different siRNAs. All siRNAs were administered at doses of 50 mg kg^{-1} in approximately 0.2 ml per injection. Measurements of apoB mRNA, apoB protein levels, lipoprotein concentrations and plasma cholesterol content were performed 24 h after the last i.v. injection. Experiments were carried out in a blinded fashion. The same experimental design was used for experiments with the human apoB transgenic mice (1004-T hemizygotes, Taconic).

In vivo bioanalytical methods

An RPA, using radiolabelled probes complementary to the antisense strands, was used to detect siRNAs in pooled liver and jejunum tissue lysates from animals treated with saline or siRNAs. RPA for endogenous miRNAs was used as a loading control for jejunum (miR-143, sequence 5′-UGAGAUGAAGCACUGUAGCUCA-3′) and liver (miR-122, 5′-UGGAGUGUGACAAUGGUGUUUG-3′).

The QuantiGene assay (Genospectra) was used to quantify the reduction of mouse apoB mRNA in liver and jejunum tissue after siRNA treatment. Small uniform tissue samples where collected 24 h after the last injection. Lysates from three tissue samples per animal were directly used for apoB and GAPDH mRNA quantification, and the ratio of apoB and GAPDH mRNA was calculated and expressed as a group average relative to the saline control group. Specific probes for detection of apoB mRNA levels were designed to the following regions of the apoB mRNA ORF: probe set 1 83–385; probe set II 5,045–5,673; probe set III 12,004–12,411. Furthermore, apoB mRNA reduction in liver was quantified from purified (RNeasy mRNA isolation kit, Qiagen), pooled mRNA for each treatment group. As well as GAPDH, factor VII, glucose-6-phosphatase and VEGF mRNAs were also used for normalization.

ELISA was used to quantify the reduction of apoB-100 protein levels in mouse plasma after siRNA treatment. apoB-100 from plasma samples of individual animals was detected using the primary antibody LF3 against mouse apoB-100 (gift of S. Young; see ref. [21]). Levels were normalized to plasma volume and expressed as group averages relative to the saline control group.

Total cholesterol levels in the plasma were measured using the Cholesterol detection kit (Diasys). For NMR determination of the plasma lipoprotein profile a Bruker DRX 600 with cryoprobe head was used (LipoFIT Analytic GmbH). Single measurements of 500 μl mouse plasma (pooled from ten animals per treatment group) were performed. The lipoprotein subclass distribution was calculated from the NMR data by using computer algorithms that are based on human blood standards[22]. The particle number for lipoprotein classes was calculated based on the correlation of known particle size and composition with the experimentally determined NMR signal intensity. On the basis of this correlation, the cholesterol content in the LDL fraction was computed. The cholesterol values calculated from NMR data were confirmed by the presence of comparable levels of total cholesterol in plasma and HDL-cholesterol as determined by enzymatic assays.

5′-RACE analysis

Total RNA (5 μg) from pooled liver and jejunum samples from animals treated with different siRNAs was ligated to a GeneRacer adaptor (Invitrogen) without prior treatment. Ligated RNA was reverse transcribed using a gene-specific primer (GSP: 5′-CTCCTG TTGCAGTAGAGTGCAGCT-3′). To detect cleavage products, PCR was performed using primers complementary to the RNA adaptor (GR5′: 5′-CTCTAGAGGCATGGACGCG AGGACACTA-3′) and apoB mRNA (Rev2: 5′-ACGCGTCGAGGTGGAGACATGAGGT TGGCATTGTTC-3′). Amplification products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The identity of specific PCR products was confirmed by sequencing of the excised bands.

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EXHIBIT 16

LETTERS

RNAi-mediated gene silencing in non-human primates

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The opportunity to harness the RNA interference (RNAi) pathway to silence disease-causing genes holds great promise for the development of therapeutics directed against targets that are otherwise not addressable with current medicines[1,2]. Although there are numerous examples of *in vivo* silencing of target genes after local delivery of small interfering RNAs (siRNAs)[3–5], there remain only a few reports of RNAi-mediated silencing in response to systemic delivery of siRNA[6–8], and there are no reports of systemic efficacy in non-rodent species. Here we show that siRNAs, when delivered systemically in a liposomal formulation, can silence the disease target apolipoprotein B (ApoB) in non-human primates. *APOB*-specific siRNAs were encapsulated in stable nucleic acid lipid particles (SNALP) and administered by intravenous injection to cynomolgus monkeys at doses of 1 or 2.5 mg kg^{-1}. A single siRNA injection resulted in dose-dependent silencing of *APOB* messenger RNA expression in the liver 48 h after administration, with maximal silencing of >90%. This silencing effect occurred as a result of *APOB* mRNA cleavage at precisely the site predicted for the RNAi mechanism. Significant reductions in ApoB protein, serum cholesterol and low-density lipoprotein levels were observed as early as 24 h after treatment and lasted for 11 days at the highest siRNA dose, thus demonstrating an immediate, potent and lasting biological effect of siRNA treatment. Our findings show clinically relevant RNAi-mediated gene silencing in non-human primates, supporting RNAi therapeutics as a potential new class of drugs.

ApoB is expressed predominantly in the liver and jejunum, and is an essential protein for the assembly and secretion of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), which are required for the transport and metabolism of cholesterol[9]. As a large, lipid-associated protein, ApoB is not accessible to targeting with conventional therapies, but it is a highly relevant and validated disease target. Elevated ApoB and LDL levels are correlated with increased risk of coronary artery disease, and inadequate control of LDL–cholesterol after acute coronary syndromes results in increased risk of recurrent cardiac events or death[10,11]. Approaches targeting ApoB with second-generation antisense oligonucleotides have progressed to pre-clinical and clinical studies[12]. Despite progress in the management of hypercholesterolaemia using HMG-CoA reductase inhibitors and other drugs that affect dietary cholesterol, there remains a significant need for new therapeutic approaches.

We have previously demonstrated silencing of *Apob* in rodents using cholesterol-conjugated siRNAs[6]. In the current study, we used a liposomal formulation of SNALP to evaluate systemic delivery of siRNA directed towards *APOB*. Preliminary evaluations were conducted in mice. Whereas administration of the *Apob*-specific siRNA siApoB-1, without formulation or chemical conjugation, at doses higher than 50 mg kg^{-1} was previously shown to have no

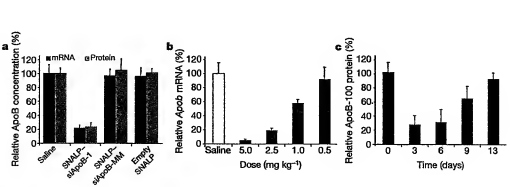

Figure 1 | SNALP–siRNA-mediated silencing of murine *Apob* is potent, specific, dose-dependent and long-lasting. a, Liver *Apob* mRNA levels normalized to *Gapdh* mRNA and serum ApoB-100 protein levels measured two days after single i.v. injections of saline, SNALP–siApoB-1 (1 mg kg^{-1}), mismatched SNALP–siApoB-MM (1 mg kg^{-1}) or empty SNALP vesicles (25 mg kg^{-1}) ($n = 5$ per group). **b**, Liver *Apob* mRNA levels normalized to *Gapdh* mRNA, assessed three days after i.v. administration of saline or 5, 2.5, 1 or 0.5 mg kg^{-1} SNALP–siApoB-2 ($n = 4$ per group). **c**, Serum ApoB-100 levels after i.v. administration of either saline or 2.5 mg kg^{-1} SNALP–siApoB-2 ($n = 6$ per group). Serum ApoB-100 levels for SNALP–siApoB-2-treated animals are relative to the saline-treated group for the same time point. Data show mean ± s.d.

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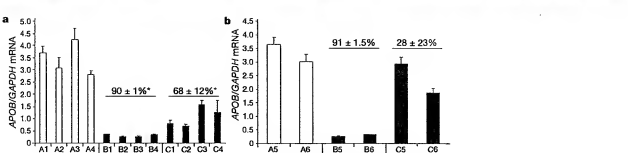

Figure 2 | Systemic silencing of *APOB* mRNA in non-human primates. **a**, **b**, Liver *APOB* mRNA levels for 12 biopsies (three isolated from each of four liver lobes) were quantified relative to *GAPDH* mRNA either 48 h (**a**, $n = 4$ animals per group) or 11 days (**b**, $n = 2$) after treatment with SNALP–siApoB-2. Data shown are mean *APOB*/*GAPDH* mRNA levels ± s.d. for each animal. Mean values (±s.d.) of the per cent *APOB* mRNA reduction relative to the saline treatment group are shown above each group. Asterisks indicate statistical significance compared with the saline-treated group ($P < 0.005$; ANOVA).

in vivo silencing activity[6], ~80% silencing of liver *Apob* mRNA and ApoB-100 protein was achieved with a single 1 mg kg^{-1} dose of SNALP-formulated siApoB-1 (Fig. 1a). In contrast, no detectable reduction was observed with a SNALP-formulated mismatched siRNA (siApoB-MM) or empty SNALP vesicles, indicating that silencing is specific to the siRNA and is not caused by the liposomal carrier. This silencing effect of SNALP-formulated siRNA represents more than a 100-fold improvement in potency compared with systemic administration of cholesterol-conjugated siApoB-1 (chol–siApoB-1) (Supplementary Fig. 1). Moreover, liposomal formulation of siRNA seems to be a general strategy for silencing hepatocyte targets, as demonstrated in mice for coagulation factor VII, green fluorescent protein and cyclophilin B (A.A., R. Constien and M.N.F., unpublished results).

As siApoB-1 was originally designed to be cross-reactive to both mouse and human ApoB genes, and we planned to conduct RNAi studies in non-human primates, a second ApoB-specific siRNA, siApoB-2, was designed to be cross-reactive with mouse, human and cynomolgus monkey ApoB genes. siApoB-2 was also selected on the basis of *in vitro* gene silencing activity and the absence of immunostimulatory activity (data not shown). Murine studies showed that encapsulated siApoB-2 showed a dose-dependent reduction in *Apob* mRNA, with >90% silencing achieved at the highest (5 mg kg^{-1}) dose (Fig. 1b). After a single 2.5 mg kg^{-1} dose of SNALP–siApoB-2, 80% silencing of liver *Apob* mRNA was associated with a 72% reduction in serum ApoB-100 protein. The silencing effect was detected for up to nine days, and was followed by recovery to normal protein levels by day 13 after treatment (Fig. 1c).

To address the therapeutic potential of this systemic RNAi approach, we evaluated the pharmacokinetics, efficacy and safety of SNALP-formulated siApoB-2 in cynomolgus monkeys. We first determined the circulating half-life of SNALP–siApoB-2 in plasma samples collected from cynomolgus monkeys ($n = 2$) receiving a single 2.5 mg kg^{-1} intravenous (i.v.) injection of the siRNA. An elimination half-life of 72 min was measured for the siRNA (Supplementary Fig. 2), compared with a 38-min half-life in mice (Supplementary Fig. 3a).

To evaluate efficacy, cynomolgus monkeys were treated with saline or SNALP-formulated siApoB-2 at doses of 1 or 2.5 mg kg^{-1} ($n = 6$ per group). siApoB-2 treatment was associated with a clear and statistically significant dose-dependent gene-silencing effect on cynomolgus liver *APOB* mRNA. Forty-eight hours after treatment, *APOB* mRNA was reduced by 68 ± 12% (mean ± s.d., $n = 4$, $P = 0.004$) and 90 ± 1% ($n = 4$, $P = 0.002$) for the 1 mg kg^{-1} and 2.5 mg kg^{-1} groups, respectively (Fig. 2a). Gene silencing was found to be consistent across the liver and correlated with detectable tissue levels of siApoB-2 (Supplementary Fig. 4). We also confirmed this *APOB* mRNA silencing to be mediated by RNAi, as demonstrated by 5′ rapid amplification of cDNA ends (RACE) analysis and identification of the predicted cleavage site, exactly ten nucleotides from the 5′ end of the antisense strand of siApoB-2 (Supplementary Fig. 5). Notably, *APOB* mRNA silencing was maintained for 11 days after the single 2.5 mg kg^{-1} treatment, with *APOB* mRNA levels still reduced by 91 ± 1.5% (Fig. 2b). Monkeys treated with the 1 mg kg^{-1} dose showed varying degrees of recovery from ApoB silencing at the day 11 time point. Although *APOB* mRNA was efficiently silenced in the liver, SNALP–siApoB-2 showed no silencing of *APOB* expressed in the jejunum (Supplementary Fig. 6), consistent with the absence of significant biodistribution of SNALP-formulated siRNAs to intestinal tissues in mice (Supplementary Fig. 3b).

The degree and persistence of RNAi-mediated silencing observed in cynomolgus monkeys far exceeds the results obtained with rodents. The lasting RNAi-mediated effects *in vivo* are consistent with observed long-lasting silencing by siRNAs in other studies[13,14], and the longer duration observed in primates may relate to species differences in the efficiency and stability of the RNA-induced

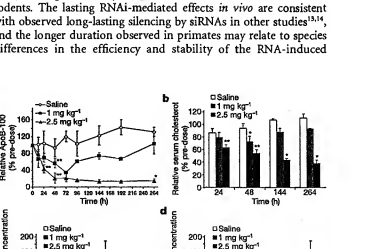

Figure 3 | Phenotypic effects of RNAi-mediated silencing of *APOB* mRNA in non-human primates. **a**–**d**, Serial plasma samples were obtained from cynomolgus monkeys treated with saline or 1 or 2.5 mg kg^{-1} SNALP–siApoB-2, and measured for ApoB-100 (**a**), total serum cholesterol (**b**), LDL (**c**) and HDL (**d**) levels. Data show levels as a percentage of pre-dose values and are expressed as mean ± s.d. Data sets collected at 0, 12, 24 and 48 h have a group size of six, and data sets collected at later time points have a group size of two. Data points marked with asterisks are statistically significant compared with saline-treated animals (*$P < 0.05$, **$P < 0.005$; ANOVA).

silencing complex (RISC), the mitotic state of hepatocytes and/or the tissue stability of the siRNA.

The expected biological effects resulting from *APOB* mRNA silencing include reduction in the blood levels of ApoB-100 protein, total cholesterol and LDL. To evaluate the kinetics of these downstream effects, we analysed plasma sampled serially from individual monkeys before and during the 11-day time course of the single-dose siApoB-2 study. Plasma ApoB-100 protein levels were reduced as early as 12 h after administration of 1 or 2.5 mg kg^{-1} SNALP–siApoB-2, reaching nadirs of 35 ± 2% and 22 ± 9% of pre-treatment levels, respectively, 72 h after treatment (Fig. 3a). Animals that received the higher siRNA dose maintained a marked reduction in ApoB protein between 2 and 11 days after treatment, consistent with the lasting effect on mRNA silencing. Monkeys that received the lower siRNA dose showed an intermediate degree of ApoB protein reduction that returned to pre-dose levels by day 11, consistent with the observed recovery in *APOB* mRNA.

Serum cholesterol levels were similarly reduced, in a dose-dependent manner and with comparable kinetics (Fig. 3b). The maximum cholesterol reduction of 62 ± 5.5% ($n = 2$, $P = 0.006$) observed for the high dose siRNA group would be considered clinically significant for patients with hypercholesterolaemia, and exceeds levels of cholesterol reduction reported clinically for currently approved cholesterol-lowering drugs.

Administration of SNALP–siApoB-2 also resulted in dramatic and rapid dose-dependent reduction in the ApoB-containing lipoprotein particle LDL. Reduction in LDL relative to pre-dose levels was observed as early as 24 h after treatment for both doses of SNALP–siApoB-2 (Fig. 3c). In contrast, there were no significant changes in circulating levels of the non-ApoB-containing high-density lipoprotein particle (HDL, Fig. 3d). The reduction in LDL persisted over the 11-day study for both siApoB-2 treatment groups, with a maximum 82 ± 7% decrease compared to pre-treatment levels observed for the high-dose group at day 11 ($n = 2$, $P = 0.003$). The time required for the biological effects to return to pre-dose levels was not determined for the high-dose group because the endpoint for this study was defined using rodent data, which indicated a faster rate of recovery. The rapid onset and lasting effect on lipoprotein metabolism suggest that siRNAs targeting *APOB* may be a valuable therapeutic strategy for achieving plaque stabilization in acute coronary syndromes[10,11], as HMG-CoA reductase inhibitors can require up to 4–6 weeks to have the desired clinical effects[15].

An important consideration for the therapeutic application of siRNA relates to its general safety, as well as to the safety profile associated with specific delivery technologies. General tolerability as well as specific toxicities (such as activation of complement, coagulation and cytokines) were evaluated for all monkeys in this study. We observed no treatment-related effects on the appearance or behaviour of animals treated with SNALP–siApoB-2 compared with saline-treated animals. There was no evidence for complement activation, delayed coagulation, pro-inflammatory cytokine production (Supplementary Table 1) or changes in haematology parameters (data not shown), toxicities that have been observed previously with treatments using related approaches[16–19]. Across a systematic evaluation, the only detected change in primates treated with SNALP–siApoB-2 was a transient increase in liver enzymes in monkeys that received the high dose of SNALP–siApoB-2. The observed transaminosis peaked 48 h after treatment and was highly variable across individual animals. These effects, which were observed only at the highest dose of SNALP–siApoB-2, were completely reversible, with normalization by day 6 notwithstanding continued biological efficacy.

Our study highlights the potential for therapeutic gene silencing using systemic RNAi in non-human primates. A single, low dose of *APOB*-specific siRNA resulted in rapid and lasting RNAi-mediated gene silencing, with associated and profound phenotypic changes. The study was limited by the premature termination of the protocol after 11 days, which prevented full evaluation of the time course for RNAi-mediated effects. Although further optimization of treatment regimen and safety profile characterization may be required, our data suggest that systemic delivery of siRNAs for targeting hepatocyte-specific genes in a higher species is possible. Furthermore, the rapid and long-lasting silencing of *APOB* using RNAi may represent a new strategy for reducing LDL–cholesterol in several relevant clinical settings.

METHODS

Additional details of the methods used are provided in the Supplementary Information.

siRNA formulation. The SNALP formulation contained the lipids 3-*N*-[(ω-methoxypoly(ethylene glycol)$_{2000}$)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar per cent ratio.

***In vivo* experiments.** Saline and siRNA preparations were administered by tail vein injection under normal pressure and low volume (0.01 ml g^{-1}) for all rodent experiments. Cynomolgus monkeys ($n = 6$ per group) received either 2 ml kg^{-1} phosphate buffered saline or 1 or 2.5 mg kg^{-1} SNALP–siApoB-2 at a dose volume of 1.25 ml kg^{-1} as bolus i.v. injections via the saphenous vein. For mRNA measurements, three liver biopsies per lobe were collected 48 h ($n = 4$) or 264 h ($n = 2$) after siRNA administration.

Bioanalytical methods. The QuantiGene assay (Genospectra) was used to quantify reduction in *APOB* mRNA levels relative to the housekeeping gene *GAPDH* in lysates prepared from mouse liver or cynomolgus monkey liver and jejunum as previously described[6] but with minor variations. Mouse[6] and cynomolgus monkey ApoB-100 protein levels were quantified by enzyme-linked immunosorbent assay (ELISA). LDL and HDL lipoprotein content were determined for plasma samples (250 μl) as described previously[6].

Statistical analysis. *P*-values were calculated for comparison of SNALP–siApoB-2-treated animals with saline-treated animals using analysis of variance (ANOVA, two-factor without replication) with an alpha value of 0.05. *P*-values less than 0.05 were considered significant.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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EXHIBIT 17

MAJOR ARTICLE

Postexposure Protection of Guinea Pigs against a Lethal Ebola Virus Challenge Is Conferred by RNA Interference

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Background. Ebola virus (EBOV) infection causes a frequently fatal hemorrhagic fever (HF) that is refractory to treatment with currently available antiviral therapeutics. RNA interference represents a powerful, naturally occurring biological strategy for the inhibition of gene expression and has demonstrated utility in the inhibition of viral replication. Here, we describe the development of a potential therapy for EBOV infection that is based on small interfering RNAs (siRNAs).

Methods. Four siRNAs targeting the polymerase (L) gene of the Zaire species of EBOV (ZEBOV) were either complexed with polyethylenimine (PEI) or formulated in stable nucleic acid–lipid particles (SNALPs). Guinea pigs were treated with these siRNAs either before or after lethal ZEBOV challenge.

Results. Treatment of guinea pigs with a pool of the L gene–specific siRNAs delivered by PEI polyplexes reduced plasma viremia levels and partially protected the animals from death when administered shortly before the ZEBOV challenge. Evaluation of the same pool of siRNAs delivered using SNALPs proved that this system was more efficacious, as it completely protected guinea pigs against viremia and death when administered shortly after the ZEBOV challenge. Additional experiments showed that 1 of the 4 siRNAs alone could completely protect guinea pigs from a lethal ZEBOV challenge.

Conclusions. Further development of this technology has the potential to yield effective treatments for EBOV HF as well as for diseases caused by other agents that are considered to be biological threats.

Ebola virus (EBOV; family *Filoviridae*) is a single-stranded, negative-sense RNA virus that is among the best known of the viruses that cause hemorrhagic fever (HF). Although outbreaks have been sporadic and geographically restricted to areas of Central Africa, the HFs caused by these viruses are remarkably severe and are associated with high case fatality rates, which often exceed 80%. In addition to causing human infection, these viruses have decimated populations of wild apes in Central Africa [1]. Although significant advances have been made in the development of vaccines to combat EBOV infection, there are at present no vaccines or effective therapies available for human use. There is clearly a need to develop effective treatments that can be used to respond to outbreaks of EBOV in Africa and to counter acts of bioterrorism that may occur. In addition, the unfortunate death of a Russian scientist after accidental exposure to EBOV [2] underscores the need for medical countermeasures for postexposure prophylaxis.

EBOV particles contain an ~19-kb noninfectious RNA genome that encodes 7 structural proteins and 1 nonstructural protein, with a gene order of 3′ leader, nucleoprotein (NP), virion protein (VP) 35, VP40, gly-

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The opinions, interpretations, conclusions, and recommendations expressed in this article are those of the authors and are not necessarily endorsed by the US Army.

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The Journal of Infectious Diseases 2006; 193:1650–7

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coprotein, VP30, VP24, polymerase (L) protein, and 5′ trailer [3]. Four of these proteins are associated with the viral genomic RNA in the ribonucleoprotein complex: NP, VP30, VP35, and the L protein. The L and VP35 proteins together comprise the polymerase complex, which is responsible for transcribing and replicating the EBOV genome. The L protein provides the RNA-dependent RNA polymerase activity of the complex and, thus, is an ideal target for antiviral approaches—not only because its suppression should lead to a nearly total loss of all RNA synthesis, but also because of the absence of similar proteins in mammalian cells.

RNA interference (RNAi) represents a powerful, naturally occurring biological strategy for inhibiting gene expression. RNAi has been used in cell-culture systems to inhibit the replication of a number of viruses that cause disease in humans, including HIV, hepatitis B virus (HBV), hepatitis C virus, influenza virus, herpesviruses, poliovirus, human papillomavirus, respiratory syncytial virus, and coxsackievirus (reviewed in [4, 5]); more recently, it has been used to inhibit some emerging and reemerging viruses, including Marburg virus [6], lymphocytic choriomeningitis virus [7], and severe acute respiratory syndrome coronavirus [8]. Although these in vitro results have been highly encouraging, the difficulty involved in the effective delivery of small interfering RNAs (siRNAs) in vivo has been the major obstacle to their use as therapeutic agents.

Several approaches have been employed in attempts to develop an in vivo siRNA delivery system. Early proof-of-concept studies included the use of mouse models and rapid hydrodynamic intravenous (iv) injection of large volumes of siRNA solution [9–11]. However, this invasive procedure appears to have little utility for human use. Several key breakthroughs have highlighted the feasibility of the use of siRNAs as antiviral therapeutics. Researchers have shown that cationic polymers have the ability to promote the successful delivery of siRNA by iv administration in influenza virus–infected mice [12]. More recently, the efficacy of lipid-encapsulated siRNAs targeted to HBV has been demonstrated in an in vivo mouse model of HBV replication [13]. In that study, siRNA targeted to HBV RNA was incorporated into a specialized liposome to form a stable nucleic acid–lipid particle (SNALP) and administered to HBV-infected mice. Importantly, the reduction in the quantity of HBV DNA observed was specific and lasted for up to 7 days after administration. Here, we used SNALPs as a postexposure treatment in a lethal animal model of EBOV HF.

MATERIALS AND METHODS

Guinea pigs and virus. An animal model of EBOV infection and pathogenesis has been developed in inbred strain 13 guinea pigs (US Army Medical Research Institute of Infectious Diseases) by serial passage of the Zaire species of EBOV (ZEBOV; Mayinga isolate) 4 times [14]. The resulting guinea pig–adapted strain gives rise to high plasma viremia (typically $>1 \times 10^5$ pfu/mL) and is highly lethal to guinea pigs, typically causing death 7–12 days after challenge. Infection experiments were performed under biosafety level (BSL)–4 biocontainment. Research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and to experiments involving animals and adhered to the principles stated in the *Guide for the Care and Use of Laboratory Animals* [15]. The BSL-4 facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Noninfectious mouse and guinea pig clearance and biodistribution experiments were conducted at Protiva Biotherapeutics, in accordance with the guidelines of the Canadian Council on Animal Care.

siRNAs. siRNAs were designed to target individual regions of the ZEBOV L gene, in accordance with "the Tuschl rules" (available at: http://www.rockefeller.edu/labheads/tuschl/sirna.html). The siRNAs duplexes were chemically synthesized by Dharmacon or TriLink Biotechnologies. Sequences used were designated as follows: EK1, 5′-GUACGAAGCUGUAUAUAAAdTdT-3′ (sense) and 5′-UUUAUAUACAGCUUCGUACdTdT-3′ (antisense); EK2, 5′-GGAUCUUGGUACAGUGUUAdTdT-3′ (sense) and 5′-UAACACUGUACCAAGAUCCdTdT-3′ (antisense); EK3, 5′-CAGGCUUAUUCCAGUUAAAdTdT-3′ (sense) and 5′-UUUAACUGGAAUAACCUGdTdT-3′ (antisense); EK4, 5′-GUAAACGGCUGAACAUUAUdTdT-3′ (sense) and 5′-AUAAUGUUCAGCCGUUUACdTdT-3′ (antisense); and EbL-Scram1, 5′-CAAAAAAAUUUUUACGGGGdTdT-3′ (sense) and 5′-CCCCGUAAAAAUUUUUUGdTdT-3′ (antisense).

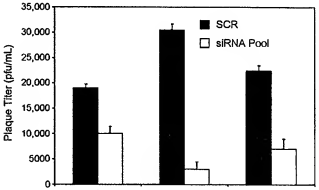

Figure 1. Inhibition of the replication of the Zaire species of Ebola virus (ZEBOV) in Vero cells by a pool of 4 different small interfering RNAs (siRNAs) targeting individual regions of the ZEBOV polymerase (L) gene. Cells were transfected with either the siRNA pool or an equivalent dose of EbL-Scram1, an irrelevant scrambled sequence (SCR), as a control. At various time points after transfection (0, 24, and 48 h), the transfected cells were infected with ZEBOV, and cells and culture fluids were harvested 24 h later, for determination of the level of infectious virus by plaque assay.

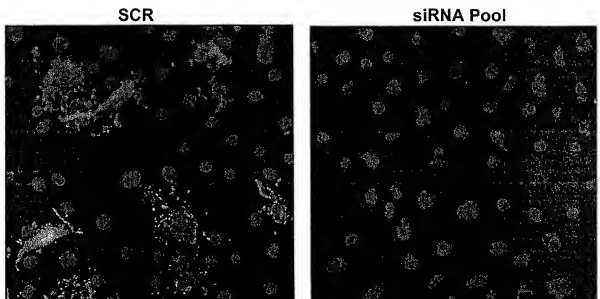

Figure 2. Inhibition of the replication of the Zaire species of Ebola virus (ZEBOV) in Vero cells, as demonstrated by immunofluorescence staining. Cells were transfected with either a pool of 4 different small interfering RNAs (siRNAs) targeting individual regions of the ZEBOV polymerase (L) gene or an equivalent dose of Ebl-Scram1, an irrelevant scrambled sequence (SCR), as a control. Cells were counterstained with 4′,6′-diamidino-2-phenylindole and Evans blue, to aid visualization. ZEBOV-positive cells are identified by green fluorescence.

Lipid encapsulation of siRNA. siRNA were encapsulated by the process of spontaneous vesicle formation reported by Jeffs et al. [16]. SNALPs were composed of synthetic cholesterol (Sigma), the phospholipid DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids), the PEG lipid PEG-C-DMA (3-*N*-[(ω-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxy-propylamine), and the cationic lipid DLinDMA (1,2-dilinoleyloxy-3-*N*,*N*-dimethylaminopropane), at the molar ratio 48:20:2:30. PEG-C-DMA and DLinDMA were synthesized as described elsewhere [17]. The resulting SNALPs were dialyzed in PBS and filter sterilized (0.2-μm filter) before use. Particle sizes ranged from 71 to 84 nm, and typically 90%–95% of the siRNA was found to be encapsulated within these liposomes.

Cell-culture experiments. siRNAs (60 pmol) were transfected into Vero cells by use of Oligofectamine (Invitrogen), in accordance with the manufacturer's instructions. At 0, 24, or 48 h after transfection, Vero cells were infected with ZEBOV at an MOI of 1.0. Culture fluids were collected at 24, 48, and 96 h for determination of the level of infectious ZEBOV, and Vero cells were collected for immunofluorescence staining.

In vivo pharmacokinetics and tissue distribution. Radiolabeled SNALPs were prepared for plasma clearance and biodistribution experiments by incorporation of 16 μCi of the nonexchangeable lipid label [^{3}H]cholesteryl oleyl ether (CHE) per milligram of total lipid [18]. SNALPs were administered in a single bolus of 0.75 mg of siRNA per kilogram of body weight to 6-week-old female and male Hartley guinea pigs (Charles River Laboratories) via ear vein injection, and blood was collected via contralateral ear nicks over the course of 24 h. At 24 h, guinea pigs were killed by CO_2 inhalation, and harvested tissues were homogenized in lysing matrix tubes (MP Biomedicals) containing 500 μL of distilled water. Homogenates were assayed for radioactivity by liquid scintillation counting with Picofluor 40 (tissues) or Picofluor 15 (blood) (PerkinElmer).

siRNA treatment and ZEBOV challenge of guinea pigs. siRNAs (30 nmol total) were mixed with polyethylenimine (PEI) (in vivo jetPEI; Qbiogene) at an N/P ratio of 5 at room temperature for 20 min, in accordance with the manufacturer's instructions. Guinea pigs were treated via intraperitoneal (ip) injection of 300 μL of the PEI polyplexes, corresponding to 8 mg/kg siRNA. Three hours after treatment, guinea pigs were challenged via subcutaneous (sc) injection of 1000 pfu of guinea pig–adapted ZEBOV. The guinea pigs received additional treatments with the PEI polyplexes (prepared as described above immediately before administration) at 24, 48, and 96 h after the ZEBOV challenge.

For experiments evaluating the SNALP delivery system, the SNALP-formulated siRNAs were administered ip 1 h after challenge of guinea pigs via sc injection of 1000 pfu of guinea pig–adapted ZEBOV. The guinea pigs received additional treatments with the SNALP-formulated siRNAs at 24, 48, 72, 96, 120, and

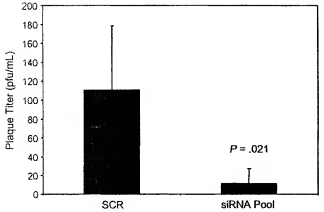

Figure 3. Plasma viremia levels of inbred strain 13 guinea pigs 4 days after challenge with the Zaire species of Ebola virus (ZEBOV). Guinea pigs were treated 3 h before the ZEBOV challenge and 1, 2, and 4 days afterward with either a pool of 4 different small interfering RNAs (siRNAs) targeting individual regions of the ZEBOV polymerase (L) gene ($n = 5$) or an equivalent dose of EbL-Scram1, an irrelevant scrambled sequence (SCR) ($n = 5$). Data are means ± SDs.

144 h after the ZEBOV challenge. The guinea pigs were carefully monitored for signs of disease and survival during the 30-day course of the experiment.

Virus titration by plaque assay. Virus titration was performed by conventional plaque assay on Vero E6 cells from cell-culture fluids of blood collected from guinea pigs, as described elsewhere [14, 19]

Immunofluorescence assay. Cells were fixed with 10% neutral-buffered formalin for 24 h, to inactivate infectious ZEBOV. After fixation, cells were washed with copious amounts of PBS and processed for immunofluorescence staining for viral proteins. Briefly, cells were incubated in ready-to-use Proteinase K (Dako) for 10 min at room temperature. Cells were then washed with PBS and blocked in normal goat serum (KPL Laboratories) for 20 min at room temperature. Viral antigen was detected by incubating cells with a mouse monoclonal antibody against the guinea pig–adapted ZEBOV for 20 min at room temperature, rinsing in PBS, and incubating with an anti-mouse–AlexaFluor 488 conjugate (Invitrogen) for 20 min at room temperature. Cells were counterstained with 4′,6′-diamidino-2-phenylindole and Evans blue, to aid visualization. The percentage of antigen-positive cells was determined by examining random fields for fluorescence.

In vivo cytokine induction. SNALPs were administered in 0.2 mL of PBS to 6–8-week-old CD1 ICR mice (Harlan) by standard iv injection in the lateral tail vein. Blood was collected by cardiac puncture 6 h after administration and then processed as plasma for cytokine analysis. Levels of the mouse cytokines interferon (IFN)–α and IFN-β were measured by use of sandwich ELISA kits (PBL Biomedical), in accordance with the manufacturer's instructions.

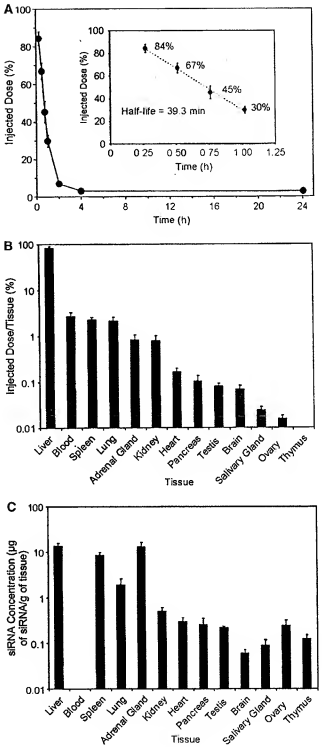

Figure 4. In vivo clearance and biodistribution of stable nucleic acid–lipid particles (SNALPs). Shown are plasma clearance *(A)* and biodistribution (*B* and *C*) of [^{3}H]cholesteryl oleyl ether–labeled SNALPs. Each guinea pig received a single intravenous injection of 0.75 mg/kg small interfering RNAs (siRNA) formulated as SNALPs. Biodistribution data were collected 24 h after injection. Data are means ± SDs (for all tissues, $n = 5$ guinea pigs, except for testes [$n = 3$] and ovaries [$n = 2$]).

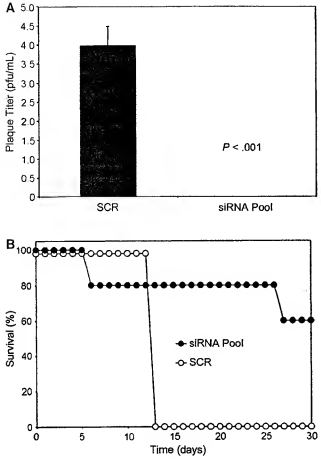

Figure 5. Antiviral efficacy of a pool of 4 different small interfering RNAs (siRNAs) targeting individual regions of the Zaire species of Ebola virus (ZEBOV) polymerase (L) gene and encapsulated in stable nucleic acid–lipid particles (SNALPs). Shown are plasma viremia levels *(A)* and survival rates *(B)* for inbred strain 13 guinea pigs after ZEBOV challenge. One hour after challenge and daily on days 1–6 thereafter, guinea pigs were treated, via the SNALP delivery system, with the siRNA pool (1.0 mg/kg) or an equivalent dose of EbL-Scram1, an irrelevant scrambled sequence (SCR). Plasma viremia levels were determined on day 7. Viremia data are means ± SDs ($n = 5$).

RESULTS

To determine whether siRNAs can inhibit the replication of ZEBOV in vitro, we first transfected Vero cells with either a pool of 4 different siRNAs (EK1–EK4) specific for the ZEBOV L gene or an equivalent dose of an irrelevant scrambled sequence (EbL-Scram1). We used Vero cells because they lack the structural genes for IFN-α and IFN-β [20–22], thus precluding any confounding effects of these cytokines. At various time points after transfection (0, 24, and 48 h), the transfected cells were infected with ZEBOV. Cells and culture fluids were harvested 24 h later, to determine the level of virus production. The results showed that the siRNA pool inhibited the production of infectious ZEBOV 2–10-fold, depending on when the transfected cells were infected (figure 1). By immunofluorescence antibody staining, we were able to demonstrate a mean ± SD reduction of 83% ± 14% ($P = .006$, Student's t test) in the numbers of cells expressing EBOV protein (figure 2). Individual testing of the 4 L gene–specific siRNAs yielded similar results (data not shown). The irrelevant scrambled sequence did not inhibit the production of infectious ZEBOV and failed to reduce the numbers of cells expressing EBOV protein (figures 1 and 2).

We then tested whether the pool of 4 siRNAs could protect guinea pigs from a lethal ZEBOV challenge. Although both mouse and guinea pig models are available for ZEBOV infection [14, 23, 24], we chose to use guinea pigs for these experiments because (1) they appear (on the basis of evaluation of coagulation changes [25, 26]) to reproduce human filoviral infection

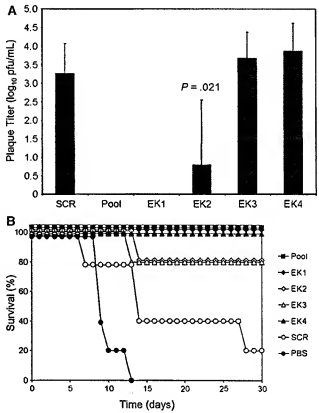

Figure 6. Antiviral efficacy of small interfering RNAs (siRNAs) targeting individual regions of the Zaire species of Ebola virus (ZEBOV) polymerase (L) gene and encapsulated in stable nucleic acid–lipid particles (SNALPs). Shown are plasma viremia levels *(A)* and survival rates *(B)* for inbred strain 13 guinea pigs after ZEBOV challenge. One hour after the challenge and daily on days 1–6 thereafter, guinea pigs were treated, via the SNALP delivery system, with a pool of 4 siRNA (0.75 mg/kg); an equivalent dose of EbL-Scram1, an irrelevant scrambled sequence (SCR); or 1 of the 4 siRNAs alone (EK1–EK4). Plasma viremia levels were determined on day 7. Viremia data are means ± SDs ($n = 5$). The P value is for the comparison between EK2 and SCR.

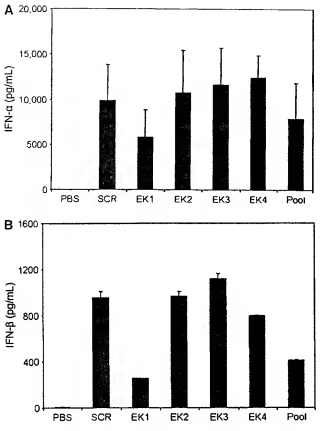

Figure 7. Small interfering RNA (siRNA)–mediated cytokine induction in mice. Shown are serum interferon (IFN)–α *(A)* and IFN-β *(B)* levels 6 h after intravenous administration of 100 μg (~5 mg/kg) of stable nucleic acid–lipid particles encapsulating either a pool of 4 different siRNAs that targeted individual regions of the Zaire species of Ebola virus polymerase (L) gene; an equivalent dose of EbL-Scram1, an irrelevant scrambled sequence (SCR); or 1 of the 4 siRNAs alone (EK1–EK4). Injection of PBS alone induced no detectable IFN-α or IFN-β. Note that injection of empty liposomes or naked siRNA alone also failed to induce detectable IFN-α or IFN-β (data not shown). Data are means ± SDs ($n = 4$).

slightly better than mice and (2) there are no mouse models available for other filoviruses, whereas guinea pigs have been used to study the Sudan species of EBOV [27] and as models for infection with several strains of the EBOV-related Marburg virus [28, 29]. In the first in vivo experiment, guinea pigs were treated with either the L gene–specific siRNA pool ($n = 5$) or the irrelevant scrambled sequence ($n = 5$), which were mixed with PEI and injected retroorbitally 3 h before the ZEBOV challenge; the guinea pigs then received equivalent doses of the siRNAs (8 mg/kg) at 24, 48, and 96 h after the ZEBOV challenge. A significant reduction in plasma viremia ($P = .02$) was demonstrated on day 4 after challenge in the guinea pigs receiving the L gene–specific siRNA pool, compared with that in the guinea pigs receiving the irrelevant scrambled sequence (figure 3). All of the control guinea pigs treated with the irrelevant scrambled sequence died or were euthanized by day 12 after challenge, whereas 1 of the guinea pigs treated with the siRNA pool survived and the other 4 guinea pigs either died or were euthanized on day 10, 13, 13, and 14, respectively.

To improve the potency of the 4 L gene–specific siRNAs, they were encapsulated in lipid particles previously shown to have antiviral efficacy in a mouse model of HBV infection [13]. The SNALP method yields particles with uniform, reproducible performance specifications regardless of the siRNA payload. The mean ± SD particle size of the 7 SNALP preparations used in the present study was 81 ± 3.0 nm, and the mean ± SD polydispersity was 0.11 ± 0.024. The mean ± SD encapsulation efficiency was 92% ± 1.5%, with a mean ± SD nucleic acid: lipid ratio of 49 ± 2.7 μg of siRNA/μmol of lipid.

To assess plasma clearance and biodistribution, SNALPs were prepared containing the nonexchangable lipid label ^{3}H-CHE [18], as described elsewhere [30]. The plasma clearance of SNALPs in guinea pigs was determined after a single iv administration. We have previously shown that iv administration of unstabilized, unformulated siRNA in mice results in rapid elimination from the plasma compartment, with an elimination half-life of ~2 min [13]. The calculated serum half-life for the SNALPs used in the present study was 39.3 min after administration in guinea pigs (figure 4*A*).

Because the liver is known to be one of the early and primary sites of ZEBOV replication in rodents and nonhuman primates [14, 31], it was also of interest to determine the pattern of biodistribution after SNALP administration. As is shown in figure 4*B* and 4*C*, substantial quantities of SNALPs accumulated in the liver (mean ± SD, 83.4% ± 6.5%) within 24 h after injection. Substantially fewer SNALPs accumulated in the spleen (mean ± SD, 2.2% ± 0.27%) and the lungs (mean ± SD, 2.29% ± 0.28%). The brain (mean ± SD, 0.068% ± 0.016%), gonadal tissues (mean ± SD, 0.016% ± 0.002% and 0.08% ± 0.012% for ovaries and testes, respectively), and thymus (mean ± SD, 0.0044% ± 0.0006%) remained relatively inaccessible, accumulating very few SNALPs. This rapid and selective accumulation of SNALPs in the liver compares favorably with the results of targeted delivery technologies that use receptor-ligand interactions as well as with the results of other targeting technologies [32–35].

We evaluated the pool of the 4 L gene–specific siRNAs using the SNALP delivery system in a rodent model of EBOV HF. As with the previous challenge experiment, 5 guinea pigs received the siRNA pool (1.0 mg/kg), and 5 control guinea pigs received the irrelevant scrambled sequence (1.0 mg/kg). However, in this experiment, the guinea pigs were not treated with siRNA before the ZEBOV challenge. Rather, treatment was initiated 1 h after the challenge, and additional treatments were administered daily on days 1–6 after infection. Plasma viremia levels, which peak on day 7 in this guinea pig model [14], were not detected on day 7 in any of the guinea pigs treated with

the siRNA pool but ranged from ~3.5 to 4.5 $\log_{10}$ pfu/mL in the control guinea pigs (figure 5*A*). One guinea pig treated with the siRNA pool died on day 6, most likely because of toxicity, given that we were unable to demonstrate the presence of infectious ZEBOV in this animal. Another guinea pig from this treatment group had to be euthanized on day 26; this death could not be attributed to viral replication either. The remaining 3 guinea pigs treated with the siRNA pool did not show any evidence of illness and survived the ZEBOV challenge, whereas all 5 of the control guinea pigs died or were euthanized by day 13 (figure 5*B*).

A further experiment was performed to assess the efficacy of the siRNA pool at a lower dose and to individually evaluate the 4 L gene–specific siRNAs (EK1–EK4). ZEBOV-infected guinea pigs that received a lower dose (0.75 mg/kg) of the siRNA pool were completely protected from viremia and death (figure 6). Although varying levels of viremia and mortality were observed among the groups treated with individual siRNAs (figure 6), the lowest levels of mortality were associated with the EK1- and EK4-treated guinea pigs.

Recent studies have demonstrated that synthetic siRNA can induce a high level of type I IFN and inflammatory cytokines in mammalian cells [36, 37] and that this immune response could contribute to the antiviral efficacy or toxicities associated with systemic administration of formulated siRNA [13, 36]. The immunostimulatory properties of the siRNA-containing SNALPs were examined directly after iv administration in mice. Strikingly, all of the SNALP-formulated siRNAs, including the irrelevant scrambled sequence, induced IFN-α and IFN-β in the serum of the injected mice (figure 7). Both the guinea pigs and mice that were treated with the immunostimulatory SNALPs showed symptoms of systemic toxicity, including a transient decrease in body weight and piloerection (data not shown), that have previously been shown to be associated with siRNA-mediated stimulation of the mammalian innate immune system [13, 36]. These adverse effects were not evident in the control animals treated with PBS or in the animals injected with empty liposomes or naked siRNA. Of note, EK1, which conferred the greatest benefit when used to treat ZEBOV-infected animals, was the least immunostimulatory of the siRNAs examined. This provides further evidence supporting an RNAi-specific antiviral effect in our infection experiments.

DISCUSSION

Currently, there are no known effective pre- or postexposure therapies for human EBOV infection. At this time, treating patients infected with EBOV consists of palliative care directed toward maintaining blood volume and electrolyte balance. A number of therapies—including IFN-α, heparin, convalescent serum, and equine anti-EBOV immunoglobulin—have been used to treat infections in humans and/or nonhuman primates, but the results have been inconsistent and, in general, of little effect [19, 38]. Although a number of postexposure treatments have shown promise in rodent models of EBOV HF [39–41], none has shown any success in nonhuman primates. To date, the only postexposure treatment that has been shown to protect nonhuman primates after challenge with ZEBOV is a strategy designed to modulate the manifestations of disease rather than to block replication of the virus; in a study in rhesus monkeys [42], we demonstrated that a postexposure strategy to mitigate the coagulation disorders that typify EBOV infection improved survival from 0% to 33%. However, improved efficacy is clearly needed.

Although there is no assurance that the SNALP-based siRNA strategy described here will protect against EBOV infection in the more-rigorous nonhuman primate models, there is reason for optimism in light of comparative data from historical studies. Specifically, as noted above, several previous studies have shown complete postexposure protection of mice [39, 40] and guinea pigs [41] against a lethal ZEBOV challenge; however, in each of these cases, the rodents were not protected against viremia. Subsequent transition of these strategies to nonhuman primates was uniformly unsuccessful in protecting macaques against a lethal infection [43, 44]. This suggests that the inability to completely inhibit viremia in rodents predicts an unfavorable outcome in nonhuman primates. In the present study, after administration of the EK1 siRNA, we were unable to detect viremia in any of the ZEBOV-challenged guinea pigs. Clearly, this observation does not guarantee success of the SNALP-based siRNA approach in nonhuman primates; on the other hand, it does offer some hope that the prospects for success are at least improved.

Here, we have focused on the L gene of ZEBOV, to demonstrate the in vivo utility of the SNALP technology. Future studies will focus on evaluating sequences of other EBOV genes and the possibility of employing cocktails of siRNAs for increased antiviral effect. Furthermore, because of the unique mechanism of RNAi, there may be benefits to combining a siRNA treatment for EBOV infection with complementary antiviral approaches, such as immunoglobulins or coagulation inhibitors.

Acknowledgments

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EXHIBIT 18

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Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle

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ABSTRACT

Potent sequence selective gene inhibition by siRNA 'targeted' therapeutics promises the ultimate level of specificity, but siRNA therapeutics is hindered by poor intracellular uptake, limited blood stability and non-specific immune stimulation. To address these problems, ligand-targeted, sterically stabilized nanoparticles have been adapted for siRNA. Self-assembling nanoparticles with siRNA were constructed with polyethyleneimine (PEI) that is PEGylated with an Arg-Gly-Asp (RGD) peptide ligand attached at the distal end of the polyethylene glycol (PEG), as a means to target tumor neovasculature expressing integrins and used to deliver siRNA inhibiting vascular endothelial growth factor receptor-2 (VEGF R2) expression and thereby tumor angiogenesis. Cell delivery and activity of PEGylated PEI was found to be siRNA sequence specific and depend on the presence of peptide ligand and could be competed by free peptide. Intravenous administration into tumor-bearing mice gave selective tumor uptake, siRNA sequence-specific inhibition of protein expression within the tumor and inhibition of both tumor angiogenesis and growth rate. The results suggest achievement of two levels of targeting: tumor tissue selective delivery via the nanoparticle ligand and gene pathway selectivity via the siRNA oligonucleotide. This opens the door for better targeted therapeutics with both tissue and gene selectivity, also to improve targeted therapies with less than ideal therapeutic targets.

INTRODUCTION

Even the emergence of 'targeted' cancer therapeutics inhibiting tumor-specific proteins or pathways, such as anti-vascular endothelial growth factor (VEGF) antibody (1), has not eliminated problems of toxicity. Thus, recognition of potent, sequence-selective gene inhibition by siRNA oligonucleotides and rapid adoption as the tool of choice in cell culture has generated the expectation for their use to improve targeted therapeutics (2–5). The prospects are for siRNA to be substantially better than antibodies, in part because they are easily applicable to virtually any therapeutic target including intracellular factors and even transcription factors. Also, they promise potent gene inhibition with exquisite selectivity, even down to the level of single-nucleotide polymorphisms, and easy identification by small screens across the gene sequence of the offending protein or variant. However, this promise is tempered by their double-stranded RNA (dsRNA) oligonucleotide nature, resembling antisense, ribozymes and gene therapy (6,7). For cancer, in particular, the pharmacological hurdles are severe. Local aqueous siRNA activity has been observed for several tissues (5,8,9), but is lacking in tumors (10), and systemic exposure can induce non-specific responses, as found for CpG DNA oligonucleotides (10,11). Thus, the 'revolutionary' potency and selectivity of siRNA inhibitors of gene expression promises to enable improved targeted cancer therapeutics, but the means for systemic administration and targeted distribution to disseminated metastatic lesions are needed.

Since administration of aqueous siRNA, even chemically stabilized, is limited by a lack of tumor activity and non-specific responses (5,8–11), the use of gene therapy vector systems is one approach. Several efforts have evaluated cationic lipids and polymers originally developed for DNA plasmids where internalization is by non-specific electrostatic interactions (12,13). For DNA, means to improve on control of cellular interactions have focused on either addition of peptide ligands for cellular receptors or addition of steric polymer coatings to inhibit non-specific interactions, and recently their combination (14–21). The resulting systems generally are perceived of having yielded good cytoplasmic delivery, but the gene expression from the plasmid remains limited by poor trafficking into the nucleus. Recent efforts achieved some success by coupling dual antibodies to the distal end of polyethylene glycol (PEG) on a sterically stabilized liposome

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by a complex procedure using post modification and incurring substantial loss of antibody and siRNA (22). Nonetheless, a need exists for relatively small peptide ligands, such as a disulfide-stabilized Arg-Gly-Asp (RGD) peptide targeting integrin expression upregulated at sites of neovasculature (23–27), and self-assembling nanoparticles without the problems of liposomes (14,21). We adapted our tissue-targeted plasmid nanoparticles (14) for siRNA, with the expectation that their activity for cytoplasmic delivery would benefit siRNA since its RNA-induced silencing complex-mediated activity occurs in the cytoplasmic compartment (4). The self-assembly of cationic polymer conjugated with PEG-peptide conjugates with siRNA was found, as with plasmid DNA, to form sterically stabilized 'layered' nanoparticle-sized polyplexes with exposed RGD ligands, referred to here as nanoplexes. These siRNA nanoplexes were evaluated with an siRNA inhibiting the VEGF-mediated pathway of tumor angiogenesis. The results of intravenous administration into tumor-bearing animals showed evidence for sequence-specific inhibition of the target gene, reduction in angiogenesis and inhibition of tumor growth.

METHODS

Nucleic acids

Short dsRNA oligonucleotides for siRNA labeled siLuc, siLacZ, siGFP and siVEGF R2 were designed based on studies by Elbashir *et al.* (2), validated to lack significant interfering homology using BLAST analysis, and synthesized and purified by Dharmacon (Lafayette, CO). Two sequences were synthesized per target and combined in a 1:1 molar ratio. The target sequences used were as follows: for siLuc: aacgcgtggagagcaactgca and aagctatgaaacgatatgggc, for siLacZ: aacagttgcgcagcctgaatg and aacttaatcgccttgcagcac, for siGFP: aagctgacccgaagttcatc and aagcagcacgacttcttccag and for siVEGF R2: aatgcggcgattggtgacagta and aagctcagcacacgaaagac [inhibition of VEGF R2 by this siRNA has been described previously (28)]. siRNA targeted against luciferase was labeled with fluorescein isothiocyanate (FITC) at the 3′ position of the sense strand with standard linkage chemical conjugation, for fluorescently activated cell sorting (FACS)-analysis and tissue distribution experiments. The luciferase-encoding pCI-Luc plasmid (pLuc) was obtained from Lofstrand Labs (Gaithersburg, MD).

Synthesis of RGD-PEG-PEI and PEG-PEI

Two PEGylated forms of branched polyethyleneimine (PEI) (P) were prepared, one with a PEG having an RGD peptide at its distal end RGD-PEG-PEI (RPP) and the other with a PEG lacking the peptide PEG-PEI (PP).

The 'cyclic' 10mer RGD peptide with the sequence H-ACRGDMFGCA-OH, was synthesized, oxidized to form an intramolecular disulfide bridge and purified to >95% purity by Advanced ChemTech (Louisville, KY). This sequence was derived from the integrin-binding RGD peptides identified by phage display and has been found effective for cell binding and internalization (23,29).

Synthesis of RPP was carried out as follows in two steps. In the first step, to a stirring solution of RGD (60 mg) in dimethyl sulfoxide (DMSO) (600 µl) TEA [8.54 µl in 20 µl of tetrahydrofuran (THF)] was added under nitrogen. After stirring for 1 min, a solution of NHS-PEG-VS (212 mg in THF: DMSO; 300 µl:100 µl) was added in one portion. The reaction mixture was stirred at room temperature for 4 h, quenched with trifluoroacetic acid (TFA) (amount equivalent to the TEA) and the mixture was lyophilized. The intermediate RGD-PEG-VS was purified by either reverse-phase high-performance liquid chromatography (HPLC) or dialysis against water, and the compound lyophilized to give a yield of 50–90%. Conjugation was confirmed by Mass Spectral analysis (matrix-assisted laser desorption ionization).

In the second step of synthesis, 100 mg (21.7 µmol) of the purified RGD-PEG-VS intermediate was dissolved in 1 ml of pure DMSO. To this solution, six equivalents of TEA dissolved in 0.5 ml THF was added and mixed. An aliquot of 9.4 mg (218 µmol in terms of amines) of PEI dissolved in dimethylformamide (0.5 ml) was added to the above solution and stirred at room temperature for 12 h. The completion of the conjugation was confirmed by the disappearance of RGD-PEG-VS on TLC. The reaction was terminated by the addition of an excess of TFA and lyophilized. The product was purified as the TFA salt by HPLC. Degree of conjugation of RGD-PEG to PEI was determined by proton NMR spectrometry on a 500 MHz spectrometer (Varian), from the ratio of the area under the peaks corresponding to the -CH_2- protons of PEI (2.8–3.1 p.p.m.) and PEG (3.3–3.6 p.p.m.). Based on this estimate, ~7% of the PEI amines were conjugated with RGD-PEG, or an average of about 40 RGD-PEG molecules attached to each 25 kDa PEI molecule reducing the average number of amines from 580 to 540 per PEI molecule. Percentage conjugation ranged from 7 to 9 for various syntheses.

Preparation of nanoplexes

Nanoplexes were prepared by mixing equal volumes of aqueous solutions of cationic polymer and nucleic acid to give a net molar excess of ionizable nitrogen (polymer) to phosphate (nucleic acid) over the range of 2 to 6. The electrostatic interactions between cationic polymers and nucleic acid resulted in the formation of polyplexes with average particle size distribution of about 100 nm, hence referred to here as nanoplexes.

Three forms of nanoplexes were prepared based on the three forms of PEI: P, PP and RPP. Earlier studies have reveled that conjugation of polycations used for DNA condensation with other macromolecules can lead to incomplete condensation and formation of structures with non-spherical morphology (30,31). Though we have not observed any of these problems with the conjugates used in this study, in order to avoid this potential problem, part of the polycation required for condensation was substituted with unconjugated PEI. Therefore, all RPP- and PP-nanoplexes contain PEI in molar equivalent to the conjugates, expressed in terms of amine concentration. These nanoplexes were thus prepared by first preparing an aqueous solution of cationic polymer containing RPP or PP with P in a 1:1 molar ratio in 5 mM Hepes buffer (pH 7). In a separate tube, nucleic acids (plasmid DNA and/or siRNA) were dissolved in the same buffer in the same total volume as the cationic polymer solution. The two solutions were then mixed together and vortexed for 30 s to make nanoplexes. Mean particle size distribution was determined with a Coulter

N4plus particle size instrument (Beckman Coulter), and ζ-potential measurements were performed on a Coulter Delsa 440 SX instrument. Both instruments were calibrated using latex beads of defined size and mobility as standards (Beckman Coulter, Miami, FL).

Cells

Human umbilical vein endothelial cells (HUVECs) (Glycotech, Rockville, MD) were cultured in E-STIM endothelial cell culture medium (BD Biosciences, Bedford, MA). N2A murine neuroblastoma cells and SVR-bag 4 murine endothelial cells (both American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Quality Biologicals, Gaithersburg, MD). The cells were cultured at 37°C in a humidified atmosphere of 5% carbon dioxide/95% air.

siRNA serum stability

Serum stability of siRNA in aqueous solution versus in nanoplex preparations was characterized using polyacrylamide gel electrophoresis. Samples of siRNA either in aqueous solution or as a RPP-nanoplex were mixed in a 1:1 ratio with fresh serum to give 50% serum concentration and incubated at 37°C. Aliquots from different incubation times of each sample were loaded onto a gel and electrophoresis performed to visualize intact siRNA. The gel conditions were performed so that aqueous siRNA would enter the gel and run as a clearly visible band when stained with ethidium bromide. Under these electrophoresis conditions, siRNA in the nanoplex remains in the loading well and is visualized by the staining.

FACS analysis of nanoplex binding to HUVEC and N2A cells

HUVEC or N2A cells growing in a nearly confluent monolayer in 6 well plates were washed with phosphate-buffered saline (PBS) and detached using 1 mM EDTA in PBS. The cells were suspended in FACS buffer (PBS supplemented with 1% BSA, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$) at 4°C, centrifuged, counted, and 1×10^5 cells were transferred to FACS tubes (BD Biosciences). The cells in 2 ml were incubated with 2 μg fluorescently labeled siRNA formulated in P- PP- or RPP-nanoplexes for 1 h at 4°C. P-, PP- and RPP-nanoplexes were made by mixing 2 μg siRNA with 0.52 μg P, (0.26 μg P + 2.4 μg PP) and (0.26 μg P + 2.9 μg RPP), respectively, to obtain complexes with N/P of 2. At the end of the incubation period, the cells were washed, fixed with 4% buffered formaldehyde and analyzed on a FACSCalibur flow cytometer (BD Biosciences). In competition experiments, the cells were preincubated with a 100-fold molar excess of unconjugated RGD peptide for 30 min at 4°C. Results were analyzed using WinMDI software version 2.8 (Joseph Trotter).

siRNA-mediated gene-silencing effects *in vitro*

The SVR-bag 4 endothelial cells, constitutively expressing β-galactosidase, were seeded in a 6 well plate with 2 ml medium per well. Medium was replaced for fresh medium without serum or antibiotics, and 10 μg of siRNA against β-galactosidase or luciferase was added either in the free form or as P- (2.6 μg), PP- (1.3 μg P + 12 μg PP) or RPP- (1.3 μg P + 14.5 μg RPP) nanoplexes. In all the cases, the siRNAs complexed to the nanoparticles were prepared as an aqueous suspension described above and added to the cells once the 2 ml of medium was replaced. After 3 h, the cells were washed with PBS and serum-containing medium was added. At 48 h after transfection, the reporter enzyme activity was detected using a β-galactosidase staining kit (Active motif LLC, Carlsbad, CA). The N2A cells were seeded in a 24 well plate with 0.5 ml of medium. When nearly confluent, the medium was replaced and they were transfected with 2 μg pLuc in Lipofectamin 2000™ (Invitrogen, Carlsbad CA) according to the manufacturer's instructions, together with 1 μg of siLuc, siLacZ or siGFP in the free form or as P-, PP- or RPP-nanoplexes. Nanoparticle solutions were prepared as above and transfection was performed as described above. The concentrations were based on the plasmid or siRNA mass given above in the final medium volume of 0.5 ml. Luciferase activity was detected after 24 h, using the luciferase assay system (Promega, Madison, WI) on a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The luciferase activity of wells treated with pLuc alone was treated as 100% and used to normalize the luciferase activity of wells treated with siRNA.

Mouse tumor model

Female nude mice (6–8 weeks of age) were obtained from Taconic (Germantown, NY), kept in filter-topped cages with standard rodent chow and water available ad libitum, and a 12 h light/dark cycle. The experiments were performed according to national regulations and approved by the local animal experiments ethical committee. Subcutaneous N2A tumors were induced by inoculation of 1×10^6 N2A cells in the flank of the mice. At a tumor volume of ~0.5–1 cm^3, mice received nanoplexes or free siRNA by i.v. injection of a solution of 0.2 ml via the tail vein. The nanoplex solutions were prepared as above, at N/P ratio of 2. For tissue distribution experiments, 40 μg fluorescently labeled siRNA was injected in the free form or as P- or RPP-nanoplexes. One hour after injection, the tissues were dissected and examined with a dissection microscope fitted for fluorescence. Microscopic examination of tissues was performed with an Olympus SZX12 fluorescence microscope equipped with digital camera and connected to a PC running MagnaFire 2.0 camera software (Optronics, Goleta, CA). Pictures were taken at equal exposure times for each tissue.

In the co-delivery experiments, plasmid and siRNA were mixed in a 1:100 molar ratio, respectively (40 μg pLuc with 13 μg siRNA), and in the sequential delivery experiments 40 μg plasmid was delivered first, followed by 40 μg siRNA 2 h later (1:300 molar ratio). The tissues were dissected, weighed and put in ice-cold reporter lysis buffer (Promega) in magnetic beads containing 2 ml tubes (Q-Biogene, Carlsbad, CA), 24 h after injection of the nanoplexes. Tissues were homogenized with a Fastprep FP120 magnetic homogenizer (Q-Biogene) and samples were assayed for reporter enzyme activity using the luciferase assay system (Promega) on a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

In the tumor growth inhibition studies, the experiment was started when the tumors became palpable at 7 days

after inoculation of the tumor cells. Treatment consisted of 40 μg siRNA per mouse in RPP-nanoplexes every 3 days intravenously via the tail vein. Tumor growth was measured at regular intervals using a digital caliper by an observer blinded to treatment allocation. Each measurement consisted of tumor diameter in two directions of ~90° apart. Tumor volume was calculated as, 0.52 × longest diameter × shortest diameter^2 (32). At the end of the experiment, the animals were sacrificed and tumor tissue and surrounding skin was excised and put on a microscopy glass slide. Tissue examination for vascularization and angiogenesis was performed by microscopy using the Olympus microscope and camera equipment described above for fluorescent tissue measurements. Tissue was trans-illuminated to visualize blood vessels in the skin and a digital image was taken and stored as described above. Tissue was snap frozen immediately thereafter for western blotting.

Western blotting

Murine VEGF R2 expression in tumor samples was detected by western blotting. Tumor tissue was put into lysing Matrix D (Bio-Rad, Cambridge, MA) together with M-Per mammalian protein extraction reagent (Pierce). Tissue was homogenized, centrifuged and supernatants collected. Equivalent amounts of extracted protein (50 μg) were mixed with sample buffer containing 5% 2-mercaptoethanol (Bio-Rad), boiled, cooled and loaded in each lane of a 6% polyacrylamide gel. Electrophoresis was performed at 30 mA and subsequently proteins were transferred to an Immunoblot polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked overnight with 3% gelatin in Tris-buffered saline (TBS). Subsequently, membranes were transferred to 1% gelatin in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20) incubated with 1 μg monoclonal anti-mVEGF R2 antibody (R&D Systems) overnight. After washing twice in TTBS, goat anti-mouse immunoglobulin G peroxidase conjugate was added in 1% gelatin for 1 h, the membrane was washed twice with TTBS and subsequently once with TBS. Antibody was stained using a Bio-Rad color reagent kit for 30 min.

Statistical analyses

Luciferase expression data *in vitro* and tumor growth inhibition were analyzed, after log transformation to normalize the data, by ANOVA using Dunnett's post-test. As *in vivo* gene expression data did not follow normal distributions, data were analyzed by non-parametric Kruskal–Wallis test with Dunn's post-test to examine the significance of the differences.

RESULTS

siRNA nanoplex colloidal properties

The siRNA nanoplex was developed with a modular approach to design molecular conjugates that incorporate the three functional requirements: self-assembly, formation of a steric polymer protective surface layer and exposed ligands. To design an siRNA nanoplex, we revisited materials used originally for plasmid DNA including PEI for the polycation complexing agent, PEG for steric stabilization and peptide ligands containing an RGD motif to provide tumor selectivity due to their ability to target integrins expressed on activated endothelial cells in tumor vasculature. While peptides containing an RGD motif can bind several integrins, their specificity is determined by the flanking amino acid sequence as well as the conformation of the binding domain (33). In this study, we used a 'cyclic' RGD peptide whose integrin-binding domain is conformationally constrained by a disulfide bond. It has an identical amino acid sequence within the cyclic region of a peptide that was shown to cause cell binding and internalization by receptor-mediated pathway when expressed on filamentous phage (23,29). This peptide was shown to inhibit cell attachment to fibronectin- and vitronectin-coated plates in a sequence-specific manner. Furthermore, when coupled to an oligolysine this peptide showed receptor-mediated DNA delivery in a variety of cells, including endothelial cells (34). In order to facilitate chemical conjugation to PEG, for these studies, an alanine residue was added to each end of this peptide outside the cyclic region (23,29). The targeted siRNA nanoplexes (shown schematically in Figure 1A) were prepared by chemical synthesis of tripartite polymer conjugates (Figure 1B) with a cationic polymer, a steric polymer and a peptide ligand (RPP), followed by nanoparticle self-assembly by mixing with nucleic acid in aqueous solution (14). This RPP conjugate allows individualized optimization or chemical replacement for each functional domain.

Upon mixing purified RPP with an aqueous nucleic acid solution, the cationic domain of the conjugate binds to negatively charged nucleic acid driving self-assembly to form a nanoparticle dispersion. Studies found that stable nanoplexes could be formed at an amine (PEI) to phosphate (nucleic acid) ratio (N/P) of 2:1. Particle size and ζ-potential results at this ratio are given in Table 1. The mean size of either RPP- or PP-nanoplexes was small, between 0.07 and 0.10 μm. Particle size remained largely unchanged for 9 days, a period for which particle size was monitored. In contrast, the mean particle size of P-nanoplexes was larger, between 0.12 and 0.17 μm, and aggregated within 24 h. The ζ-potential of P-nanoplexes with siRNA was found to be highly positive, 35 ± 4 mV, as typically found with plasmid DNA, but incorporation of the PEG conjugate of PEI resulted in a reduction in the ζ-potential to 5 ± 6 mV and 6 ± 1 mV for PP-nanoplexes and RPP-nanoplexes, respectively. Surface charge polarity and amplitude depended on the ratio of the two components, but at the same ratio amplitude decreased when PEG was present (data not shown), indicating that a steric polymer layer is formed on the nanoplex surface (14,20,21).

These measurements of particle size and ζ-potential indicate that the RPP-nanoplexes formed with siRNA exhibit colloidal surface properties indicative of an outer steric polymer layer and potentially exposed RGD ligand to mediate cell-binding selectivity. This nanoplex self-assembly occurs by simple mixing of aqueous solutions of RPP conjugates with siRNA (RPP-nanoplexes). Their colloidal and biological properties were compared either to preparations with precursor conjugates lacking the RGD peptide (PP-nanoplexes) or to unconjugated PEI (P-nanoplexes).

In Vitro ligand-mediated uptake and gene inhibition

In vitro studies were performed to characterize the ligand-mediated uptake and intracellular activity of the entrapped siRNA. For use of microscopic imaging and FACS analysis,

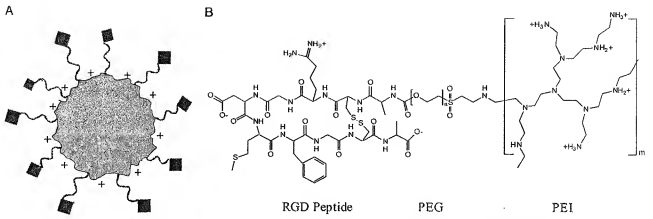

Figure 1. Design of targeted self-assembling siRNA nanoplex and activity *in vitro*. (A) Schematic structure of the targeted self-assembling nanoplex. Electrostatic interactions between negatively charged siRNA and cationic polymer result in the formation of a complex (speckled particle) that is protected by exposed steric polymer strands (wavy lines), and which gains target selectivity through a targeting ligand moiety coupled to the distal end of the steric polymer (diamonds). (B) Schematic structure of the RPP-polymer. The polymer is designed with the three functional domains needed to obtain the targeted self-assembling nanoplex as described in (A). Branched PEI with a molecular weight of ~25 kDa (~581 monomer units) is used as the cationic complexing polymer and contains on average 25% primary, 50% secondary and 25% tertiary amines. Approximately 7% of the amines are modified with PEG with an average molecular weight of 3.4 kDa (80 monomer units). The PEG is conjugated to a folded RGD peptide containing a disulfide bridge between two cysteine residues, a ligand for integrins.

Table 1. Particle characterization of siRNA nanoplexes[a]

siRNA nanoplex	Mean particle size (μm)	ζ-potential (mV)
PEI	0.12 ± .04	35 ± 4
PP	0.09 ± .01	5 ± 6
RPP	0.09 ± .01	6 ± 1

[a]amine:phosphate ratio of 2:1, average values of three preparations.

these studies relied on standard, commercially available FITC conjugates of siRNA. Initially, some stability studies were performed to characterize lability of the siRNA oligonucleotides in 50% serum in aqueous solution and in the nanoplex. The results, shown in Figure 2, demonstrate that serum incubation of siRNA oligonucleotides in aqueous solution resulted in degradation of the oligonucleotide (loss of band in the gel) after several hours, but siRNA in the nanoplex was protected for the entire length of the studies, 12 h. Additionally, microscopy studies of tumor cell transfection in culture using FITC-siRNA showed that as found by the siRNA supplier, this standard FITC conjugate did not interfere with siRNA gene inhibition activity and FITC fluorescence in cells correlated with loss of reporter gene expression (data not shown). These results support cell culture and animal administration where the siRNA is exposed to serum nucleases, at least for periods up to a few hours and for longer periods in the case of siRNA nanoplexes.

FACS analysis of endothelial (Figure 3A) and tumor cell (Figure 3B) binding studies using FITC-labeled siRNA shows that in the absence of ligand, the steric polymer surface layer reduces non-specific cell binding by 6- to 8-fold (P-nanoplexes versus PP-nanoplexes in Figure 3A and B), correlating with a reduction in ζ-potential. The presence of the peptide restores binding in a ligand-specific manner (RPP-nanoplexes in Figure 3A and B), despite maintenance of a near neutral surface charge. This binding is inhibited by the addition of unconjugated RGD peptide via competitive binding (closed bars in Figure 3A and B). These observations show that the RPP-nanoplex has both a PEG surface layer that provides steric inhibition of non-specific electrostatic cell binding and, more importantly, an RGD ligand-mediated cell binding, presumably through integrins expressed on both cell types studied (25,35).

Gene-silencing activity of the preparations was determined with cell culture assays of reporter gene-targeted siRNA using both transient and endogenous gene expression assays. Both P- and RPP-nanoplexes inhibit transient luciferase (pLuc) expression (Figure 3C) or endogenous β-galactosidase expression (Figure 3D) in a sequence-specific manner. Thus, both non-specific and ligand-mediated cellular uptake can enable sequence-specific siRNA activity in these cells. A lack of inhibition with aqueous siRNA or PP-nanoplexes reflects poor intracellular availability but for different reasons: siRNA in aqueous solution has a strong anionic character reducing intracellular uptake while in the case of PP-nanoplexes cellular uptake is diminished by the surface PEG steric layer. Thus, *in vitro* gene-silencing activity of the different siRNA nanoplex preparations is paralleled by their cell interaction and intracellular uptake.

Tumor uptake, targeted gene inhibition and phenotypic effect

Since both RPP-nanoplexes and P-nanoplexes display activity in cell culture, they were selected for *in vivo* studies in tumor-bearing mice. Studies were performed to determine whether increased tumor levels of siRNA can be achieved by siRNA nanoplexes administered by i.v. injection to tumor-bearing animals. Imaging of FITC-labeled siRNA uptake into established neuroblastoma N2A tumors in nude mice was used to observe tumor accumulation. Fluorescence microscopy to detect FITC fluorescence in tumor, lung and liver are shown in Figure 4 for animals administered aqueous siRNA,

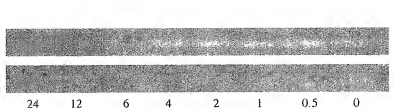

Figure 2. Degradation of siRNA in serum. Degradation of siRNA oligonucleotides when exposed to serum was measured for RPP-nanoplexes and compared with aqueous siRNA. The siRNA nanoplex, or aqueous siRNA, was incubated in 50% serum from 0 to 24 h and then the remaining intact siRNA determined by gel electrophoresis. Staining of the siRNA bound in nanoplexes shows it remains in the loading well without evidence for degradation, while the aqueous siRNA runs as a brightly stained band that diminishes with incubation time.

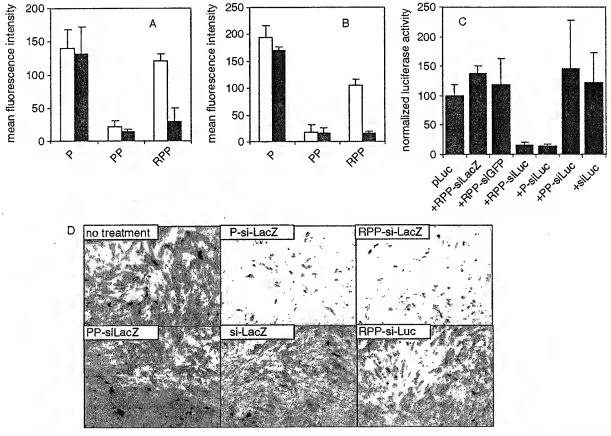

Figure 3. *In vitro* activity of siRNA nanoplex. (**A** and **B**) siRNA nanoplex binding to cells. 1×10^5 HUVEC (A, open bars) or N2A cells (B, open bars) were incubated with 2 μg fluorescently labeled siRNA formulated in P-, PP- or RPP-nanoplexes for 1 h at 4°C. Both cell types express integrins. After the incubation period, the cells were washed, fixed with 4% buffered formaldehyde and cell-bound fluorescence analyzed by FACS analysis. Unshielded positively charged P-nanoplexes showed relatively high cell binding to both cell types. Shielding of the charged nanoplex with PEG (PP-nanoplexes) reduced cell interaction, which was restored by coupling of the RGD peptide to the distal end of the PEG-shield (RPP-nanoplexes). Pre-incubation of HUVEC (A, closed bars) or N2A (B, closed bars) with a 100-fold molar excess of RGD peptide reduced binding of RPP-nanoplexes while leaving binding of P- or PP-nanoplexes unaffected, indicating that the binding of RPP-nanoplexes to cells is mediated by the RGD peptide targeting ligand. (**C**) Luciferase silencing *in vitro*. N2A cells were transfected with 2 μg luciferase plasmid, using cationic lipids without siRNA or cotransfected with 1 μg siRNA formulated as RPP-siLacZ, RPP-siGFP, RPP-siLuc, P-siLuc, PP-siLuc or free siLuc. Sequence-specific silencing of luciferase expression with siLuc is observed for RPP- and P-nanoplexes. Luciferase activity of cells treated with various agents were normalized assuming the activity of cells transfected with luciferase plasmid to be 100%. (**D**) β-Galactosidase silencing *in vitro*. SVR-bag 4 endothelial cells, constitutively expressing β-galactosidase were left untreated or incubated with 10 μg of siRNA as follows: P-siLacZ, RPP-siLacZ, PP- siLacZ, RPP-siLuc or free siLacZ. After 5 h incubation, the cells were washed and 48 h after transfection, the cells were stained for β-galactosidase activity. Only panels P-siLacZ and RPP-siLacZ show clear inhibition of β-galactosidase expression.

P-nanoplexes and RPP-nanoplexes. Intravenous administration of aqueous siRNA did not produce appreciable FITC-siRNA fluorescence in the tumor. Also, for this sample very little FITC fluorescence can be observed in the liver and even less in the lung. These results are most likely a reflection of rapid clearance of the FITC-siRNA into the urine, poor tissue accumulation except liver and potentially metabolic instability resulting in rapid excretion or liver

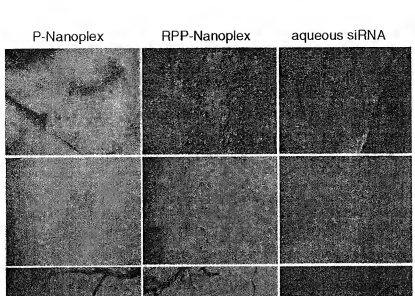

Figure 4. Tissue distribution of siRNA nanoplexes in N2A tumor-bearing mice. Mice received 40 μg fluorescently labeled siRNA by intravenous injection as P-nanoplexes (left column), or RPP-nanoplexes (middle column) or aqueous solution (right column). One hour after injection, tissues were dissected and examined on a fluorescence microscope. Pictures were taken at equal exposure times for each tissue. P-nanoplexes show punctate fluorescence in all organs, especially high in lung and liver. RPP-nanoplexes show lower fluorescence levels in lung tissue with a punctate distribution and lower non-punctate fluorescence in liver. Higher levels of fluorescence were observed in the tumor as compared with P-nanoplexes. Fluorescence levels after administration of free siRNA were much lower in all organs, as compared with either of the two nanoplex formulations.

metabolism of the FITC. The lack of tumor fluorescence from the aqueous FITC-siRNA indicates that any instability of the FITC linkage that would result in loss of FITC from nanoplex preparations will not yield tumor fluorescence (since it was not yielded by aqueous FITC-siRNA, which is more labile in serum than that in the nanoplex as shown in Figure 2 above). This conclusion is confirmed by a lack of tumor FITC fluorescence when P-nanoplexes were administered (Figure 4). This lack of tumor FITC fluorescence demonstrates that neither the P-nanoplexes accumulate in the tumor to any detectable extent nor does any FITC linkage stability in the siRNA nanoplex result in artifactual tumor fluorescence. On the other hand, the FITC-siRNA in P-nanoplexes does produce appreciable FITC-siRNA fluorescence in liver and especially in lung with a punctate profile (Figure 4). Again, this finding further supports the utility of the FITC-siRNA labeling since many plasmid studies have revealed the clearance of P-nanoparticles to distribute into these two tissues and with the punctate pattern observed here with siRNA. In contrast, RPP-nanoplexes produced appreciable FITC-siRNA fluorescence in the tumor, but poor liver and lung accumulation and a reduced punctate fluorescence pattern. This provides strong evidence that the RPP nanoplex potentially exhibits reduced non-specific tissue interactions reducing the liver and lung uptake and accumulation in tumor due to ligand binding. Also, P-nanoplexes but not RPP-nanoplexes produced occasional minor adverse effects such as piloerection, also reported to occur with plasmid DNA and attributed to non-specific charge interactions with blood components, aggregation and trapping of aggregates in tissue capillaries (21,36). Since the FITC fluorescent label is covalently attached to the siRNA with a linkage routinely used for oligonucleotides with known *in vivo* stability, the fluorescence distribution observed in these tissues likely corresponds to distribution of siRNA and not to FITC linkage instability. Importantly, the studies of siRNA serum stability in Figure 2 clearly demonstrate that the siRNA in the RPP-nanoplex is protected from degradation for several hours and is more stable than aqueous siRNA. Moreover, the fluorescent microscopy tissue distribution studies were performed after a very short time frame, 1 h, which was much shorter than the 6 h serum stability observed for aqueous siRNA and 12 h stability observed for the RPP-nanoplex. Furthermore, even administration of the FITC-conjugated siRNA in aqueous form, which is more sensitive to degradation in serum, did not result in any significant accumulation in any of the tissues measured. These results indicate that the RPP siRNA nanoplex gives increased tumor levels of siRNA molecules and led to studies of siRNA biological activity in the tumor described below.

The tumor accumulation observed with RPP-nanoplexes may occur through RGD peptide specificity for tumor endothelial cells (23–27) or by passive tumor accumulation through 'enhanced permeability and retention' effect (14,19,21,37), or both. While there are differences in these two mechanisms, the key issue is whether distribution into the tumor achieves cellular uptake and siRNA activity. This was evaluated using

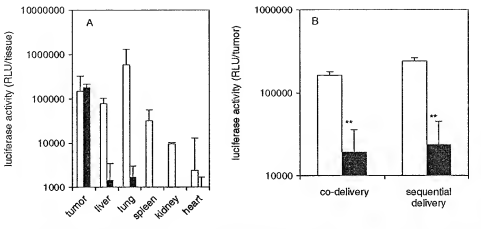

Figure 5. Inhibition of plasmid-mediated reporter gene expression *in vivo* by siRNA-nanoplexes. (A) RPP-plasmid mediated luciferase expression in tumor. N2A-tumor-bearing mice received a single intravenous injection of 40 μg pLuc in either P-nanoplexes (open bars) or RPP-nanoplexes (closed bars). After 24 h following administration, tissues were dissected, homogenized and assayed for luciferase activity. The tissues obtained from mice treated with plasmid in P-nanoplexes show no tumor-specific expression and injection was associated with side effects. Tissues obtained from mice treated with plasmid in RPP-nanoplexes displayed appreciable levels of luciferase expression only in the tumor ($n = 5$). (B) Plasmid delivery combined with siRNA delivery. N2A-tumor-bearing mice were treated (i.v. injection) with pLuc plasmid as in 5A but received either 13 μg siRNA simultaneously (co-delivery) or 40 μg siRNA 2 h later (sequential delivery) in RPP-nanoplexes. In both the cases, expression of luciferase in the tumor treated with irrelevant siRNA (open bars) was similar to the expression obtained after injection of plasmid alone (A) and treatment with Luc siRNA in RPP-nanoplexes results in ~90% gene silencing (closed bars) ($n = 5$).

transient expression of luciferase in tumor and its inhibition mediated by an siRNA targeted against luciferase mRNA. Since RPP-nanoplexes can be used for tumor-targeted delivery of siRNA as well as plasmid DNA, the experiment was carried out in two different ways, co-delivery of plasmid with siRNA or sequential delivery of plasmid followed by siRNA 2 h later, all using RPP-nanoplex. Tumor-targeted luciferase activity, achieved by the delivery of luciferase plasmid using RPP-nanoplexes to tumor-bearing mice, is shown in Figure 5A. The plasmid expression profile showing highest expression in tumor tissue parallels the tissue distribution observed with fluorescent siRNA (Figure 5A). Note that RPP-nanoplexes with plasmids also represent an attractive tissue selective gene delivery system in its own right, including constructs to express siRNA *in vivo* (R. Schiffelers, A. Ansari, C.J. Snel, M.H.A.M. Feus, G. Molema, G. Zhou, G. Strahan, G. Storm, M.C. Woodle and P.V. Scaria, manuscript in preparation). RPP-nanoplexes with siRNA inhibited plasmid expression by ~90% ($P < 0.01$) regardless of whether administered simultaneously (co-delivery) or 2 h later (sequential delivery) and was highly sequence specific (Figure 5B). These experiments clearly demonstrate that the RPP-nanoplex can be used for the delivery of siRNA to tumor tissue through i.v. administration and can provide sequence-specific inhibition of gene expression in tumor. Next, whether the siRNA delivery achieved through RPP-mediated delivery into tumor would be sufficient to provide therapeutic efficacy was tested using an siRNA targeted to an endogenous therapeutic gene. For these studies, siRNA targeting murine VEGF R2 was selected and used with RPP-nanoplexes since it is a pivotal factor in tumor angiogenesis (1,13,38). For therapeutic effects on this gene, however, the siRNA requires delivery into host (murine) endothelial cells within the tumor to elicit a phenotypic effect on tumor growth. Efficacy studies were performed with siRNA inhibiting expression of murine VEGF R2, characterized in cell culture (data not shown). Studies were performed with this therapeutic gene siRNA administered intravenously as RPP-nanoplexes every 3 days. The results, shown in Figure 6, show strong inhibition of tumor growth rate (Figure 6A) and this was sequence specific. This result suggests that the RPP siRNA nanoplex acts through an endothelial cell uptake mechanism.

Further studies were performed to obtain molecular and mechanistic evidence for mVEGF R2 siRNA gene inhibition within the tumor. Tumor angiogenesis was characterized along with VEGF R2 expression levels. Reduced tumor growth rate was paralleled by reduction in blood vessels in the vicinity immediately surrounding the tumor (Figure 6B–D). Additionally, the few blood vessels visible in the tumor treated with mVEGF R2 siRNA (Figure 6D) exhibit evidence of erratic branching expected from silencing VEGF R2 expression (1,38). Expression of VEGF R2 in treated tumors was also reduced in a sequence-specific manner (Figure 6E). Taken together, these results support the interpretation that the tumor inhibition by siVEGF R2 in RPP-nanoplexes occurs as a result of effective delivery of the siRNA into tumor vasculature producing a sequence-specific inhibition of VEGF R2 expression, tumor angiogenesis and growth.

DISCUSSION

The layered, self-assembled siRNA nanoplex shows both tissue-specific delivery and siRNA gene-specific targeting with intravenous systemic administration. Thus, this system combines tissue-targeted selectivity with the gene sequence selectivity of siRNA. The RGD peptides used here to target integrin expression on neovasculature proved effective for targeting tumor neovasculature. Nonetheless, the modular chemical design allows substitution with other ligands, or combinations of ligands, to selectively target other tissues. The modular design of this layered nanoplex provides a

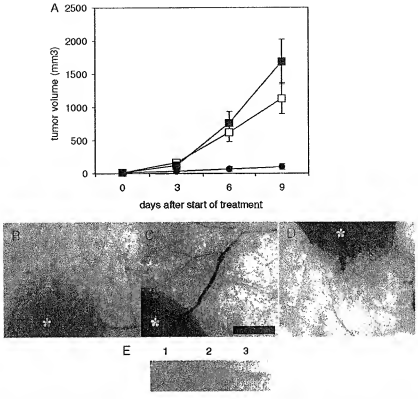

Figure 6. Tumor growth inhibition by VEGF R2 siRNA nanoplexes. (A) Tumor growth inhibition by siRNA RPP-nanoplexes. Mice were inoculated with N2A tumor cells and left untreated (open squares) or treated every 3 days by tail vein injection with RPP-nanoplexes with siLacZ (filled squares) or siVEGF R2 (filled circles) at a dose of 40 μg per mouse. Treatment was started at the time-point that the tumors became palpable (~20 mm^3). Only VEGF R2-sequence-specific siRNA inhibited tumor growth, whereas treatment with LacZ siRNA did not affect tumor growth rate as compared with untreated controls ($n = 5$). (B–D) Neovascularization in tumors treated with siRNA RPP-nanoplexes. Representative tumors excised at the end of the tumor growth inhibition experiment (A) were examined using low magnification light microscopy. Trans-illumination of tumor and surrounding skin tissue shows strong neovascularization in mice left untreated (B) and mice treated with RPP-nanoplexes with siRNA-LacZ (C). In contrast, mice treated with RPP-nanoplexes with VEGF R2 siRNA showed low neovascularization and erratic branching of blood vessels (D). Asterisks indicate tumor tissue. Bar = 2 mm. (E) VEGF R2 expression in tumor tissue after treatment with siRNA RPP-nanoplexes. Representative tumors removed at the end of the tumor growth inhibition experiment (A) were homogenized and VEGF R2-expression levels measured by western blotting. The largest tumors in the group treated with mVEGF R2 siRNA in RPP-nanoplexes and the smallest tumors in the other two groups were removed at the end of the tumor growth study 3 days following the final treatment. Lane 1 is untreated tumor, Lane 2 is VEGF R2 siRNA treated tumor and Lane 3 is LacZ siRNA treated tumor.

versatile system that can be tailored to the requirements of the physiology and to the requirements of the nucleic acid. The use of synthetic siRNA oligonucleotides compacted and protected within a nanoparticle has several potential advantages over a gene therapy approach for the expression of dsRNA RNAi agents, including adaptability to alternative chemical forms of nucleic acids and avoidance of virus safety and immunogenicity problems. Nonetheless, new chemical polymer conjugates and the nanoparticles they form face their own hurdles, but offer a much wider range of materials limited only by the scope of polymer chemistry.

The results obtained here open up new perspectives for the development of targeted cancer therapeutics with two levels of selectivity using intravenously targeted siRNA within layered nanoparticles. To utilize siRNA gene silencing of endogenous genes in a therapeutic setting, however, identification of target cell types and target genes is required, in addition to an understanding of the temporal and spatial gene-expression effect required for efficacy. Exquisite pathological tissue selectivity using a single gene, single-nucleotide polymorphism or splice variant may be possible with siRNA, but identification of such unique therapeutic targets is proving difficult. This hurdle becomes much less of a requirement for targeted therapeutics when tissue selective delivery is combined with an inhibitor of a less than ideal therapeutic target. Even more importantly, however, long before such therapeutic benefits are realized, tissue-targeted siRNA permits vast improvements in characterizing gene and RNA function in animal models of human disease that can help to identify the most selective targets for targeted therapeutics, not just siRNA. For targeted cancer therapeutics, tumor targeting of siRNA of different sequences but virtually identical physical chemistry promises to enable inhibition of multiple tumor selective pathways in one therapeutic formulation.

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EXHIBIT 19

Gene Therapy (2005) 12, 461–466

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BRIEF COMMUNICATION

RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo

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RNA interference (RNAi) represents a powerful, naturally occurring biological strategy for inhibition of gene expression. It is mediated through small interfering RNAs (siRNAs), which trigger specific mRNA degradation. In mammalian systems, however, the application of siRNAs is severely limited by the instability and poor delivery of unmodified siRNA molecules into the cells in vivo. In this study, we show that the noncovalent complexation of synthetic siRNAs with low molecular weight polyethylenimine (PEI) efficiently stabilizes siRNAs and delivers siRNAs into cells where they display full bioactivity at completely nontoxic concentrations. More importantly, in a subcutaneous mouse tumor model, the systemic (intraperitoneal, i.p.) administration of complexed, but not of naked siRNAs, leads to the delivery of the intact siRNAs into the tumors. The i.p. injection of PEI-complexed, but not of naked siRNAs targeting the c-erbB2/neu (HER-2) receptor results in a marked reduction of tumor growth through siRNA-mediated HER-2 downregulation. Hence, we establish a novel and simple system for the systemic in vivo application of siRNAs through PEI complexation as a powerful tool for future therapeutic use.
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Keywords: *RNAi; polyethylenimine; PEI; siRNA; gene targeting; HER-2*

RNAi is induced by small 21–25 nt double-stranded small interfering RNAs (siRNAs), which become incorporated into the RNA-induced silencing complex (RISC) and serve as a guide for endonucleolytic cleavage of the complementary target mRNA ([1–3] and references therein). Since siRNAs play a pivotal role in this process, the critical factors that will determine the success of RNA interference (RNAi) approaches are the ability to deliver intact siRNAs efficiently into the appropriate cells. Numerous studies describe DNA vectors, which upon viral or nonviral transfection lead to intracellular expression of double-stranded RNA (eg.[4–10]). While this approach often results in robust downregulation of gene expression *in vitro*, it suffers *in vivo* from problems similar to those of gene therapy including nonspecific effects, low transfection efficiency, poor tissue penetration, and safety concerns. Alternatively, the transfection of chemically synthesized or *in vitro* transcribed siRNAs represents a direct method of inducing RNAi *in vitro*; however, problems *in vivo* include (i) the instability and rapid degradation of the siRNAs as well as (ii) their poor cellular uptake. It is clear that the efficient delivery of intact siRNAs will play a rate-limiting role in any mammalian gene therapy application and has so far widely limited *in vivo* applications of RNAi. Although limited data exist that the *in vivo* application of noncomplexed, 'crude' siRNA might work under certain circumstances, a system stabilizing siRNAs and enhancing their delivery into cells would clearly offer advantages.

Previously, it has been shown that polyethylenimine (PEI) is able to form noncovalent interpolyelectrolyte complexes with DNA[11] and RNA.[12] On this basis, PEIs with various molecular weights, degrees of branching, and other modifications have been used as transfection reagent in a variety of cell lines and live animals to establish its efficacy for DNA delivery (for a review, see Kichler[13] and Wagner *et al* [14] and references therein). This also includes antisense oligonucleotides and siRNA *in vitro*.[15,16]

To address the question of siRNA stabilization and protection against degradation through nucleases, we performed the PEI complexation of siRNA molecules with low molecular weight PEI. In the first set of experiments, we complexed ^{32}P-end-labeled siRNAs with a commercially available linear low molecular weight PEI (JetPEI), and the complexes were incubated at 37°C in the presence of fetal calf serum (FCS). At various time points, samples were subjected to gel electrophoresis and blotted. While in the case of noncomplexed siRNAs full degradation due to RNases in the FCS was observed already at the shortest time points assayed, PEI complexation led to almost complete protection of the siRNAs against degradation as indicated by the presence of radiolabeled bands representing the full-length siRNA molecule (Figure 1a).

To test for the cellular uptake and bioactivity of PEI-complexed siRNAs, we generated clonal, stably luciferase-expressing SKOV-3 ovarian carcinoma cell lines with

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various luciferase expression levels. We preferred this system over transient cotransfection of the pooled target gene+siRNA since the targeting of a stable, endogenously expressed gene represents a more relevant model for RNAi applications. For luciferase downregulation, we used commercially available synthetic siRNA duplexes with an optimized sequence and, as negative control, an unrelated siRNA. In the absence of serum in the culture medium, addition of the specific siRNA using the established transfection reagent TransIT-TKO under transfection conditions which were optimized for DNA plasmid transfections in our lab resulted in a robust, dose-dependent downregulation of luciferase activity (Figure 1b, upper left). The application of the same transfection conditions in the presence of serum, however, led to no reduction of luciferase activity indicating that here siRNA activity was lost most likely due to siRNA degradation (Figure 1b, upper right). In contrast, when siRNAs were complexed with PEI, RNAi-mediated decrease of luciferase activity in transiently (Figure 1b, lower left) or stably (Figure 1b, lower right) luciferase expressing SKOV-3 cells was again observed also in the presence of serum. The dose-dependent downregulation was detectable already at 24 h, reached its maximum after ~48 h and was stable for several days (Figure 1b, lower panel and data not shown). This demonstrates that upon PEI-complexation, siRNA is (i) protected against degradation also in the presence of nucleases, (ii) internalized by the cells, and (iii) released intracellulary displaying full bioactivity.

To test our system *in vivo*, we chose a target gene with proven relevance in tumor therapy in man. The HER-2 (neu/c-*erb*B-2) proto-oncogene belongs to the epidermal growth factor (EGF) receptor family with heterodimeric, HER-2 containing receptor combinations showing super-

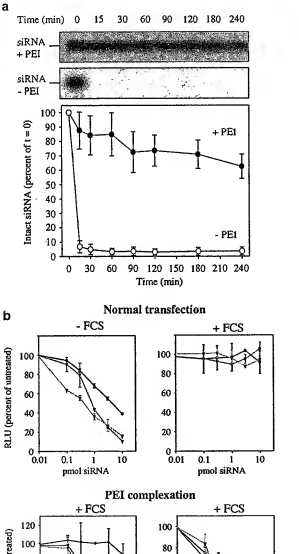

Figure 1 Stabilization and activity of siRNAs upon PEI complexation. ^{32}P-end-labeling of siRNAs was performed using T4 polynucleotide kinase according to standard protocols with 8 µg siRNA and 50 µCi γ-[^{32}P]ATP. For PEI complexation, 100 pmol specific and/or nonspecific siRNAs (Dharmacon, Lafayette, CO, USA) were dissolved in 200 µl 150 mM NaCl, pH 7.4, and incubated for 10 min. 1.25 µl 4xJetPEI (Qbiogene, Wiesbaden, Germany) was dissolved in 200 µl 150 mM NaCl, pH 7.4, and after 10 min pipetted to the siRNA solution resulting in N/P ratio = 10. After vortexing, the mixture was incubated for 1 h at room temperature, prior to use. (a) Determination of siRNA stability in vitro. ^{32}P-labeled siRNAs, complexed (closed circles) or not complexed (open circles) with PEI, were subjected to treatment with 1% fetal calf serum at 37°C. At the time points indicated, 1% sodium dodecyl sulfate was added and mixtures were heat-denatured for 5 min at 100°C. The samples were analysed by agarose gel electrophoresis and bands representing full-length siRNA molecules were quantitated. (b) PEI complexation leads to cellular delivery of bioactive siRNAs also in the presence of FCS. Clonal SKOV-3 ovarian carcinoma cell lines with stable constitutive luciferase expression were generated by transfection with a recombinant luciferase expression vector (pGL3-Basic plasmid (Promega, Madison, WI, USA) with an inserted human NF-kB promoter driving luciferase expression). Stable mass-integrants were selected using G418 at 1 mg/ml prior to the generation of clonal cell lines by limited dilution. For luciferase targeting experiments, the optimized siRNA pGL3 was used with the unrelated siRNA pGL2 serving as negative control (Dharmacon, Lafayette, CO, USA). For transfections using TransIT-TKO (Mirus, Madison, WI, USA), conditions previously determined as optimal in our lab for high plasmid transfection efficacies in 24-well plates were used: 1.125 µl of the transfection reagent was diluted in 20 µl serum-free IMDM medium and the mixture was incubated for 10 min prior to addition of a solution of 10 pmol siRNAs+0.25 µg unrelated plasmid DNA and a final incubation for 60 min. PEI-siRNA complexes were prepared as described above. Cells were transfected at ~70% confluency by addition of the PEI-complexed siRNAs or the TransIT-TKO-siRNA mixture in the presence or absence of 2% FCS as indicated. The determination of luciferase activity was performed at the time points indicated using the luciferase assay kit from Promega (Mannheim, Germany) according to the manufacturer's protocol. While in the absence of serum, standard transfection conditions (eg, TransIT-TKO, upper left) result in the robust downregulation of endogenous luciferase expression, siRNA bioactivity is lost in the presence of serum (upper right). Upon PEI complexation, however, luciferase siRNAs display targeting activity for several days in transiently (lower left) or stably luciferase expressing cells (lower right) also upon transfection in the presence of serum.

ior signal-transducing, antiapoptotic and cell growth-stimulating capabilities.[17] HER-2 overexpression has been observed in a wide variety of human cancers and cancer cell lines, and has been generally linked to an unfavorable prognosis and more aggressive malignant behavior of tumors (eg, Slamon *et al*[18]). The humanized monoclonal anti-HER-2 antibody trastuzumab (Herceptin (®)), has been approved for the adjuvant treatment of advanced breast and ovarian cancer (see McKeage and Perry[19] for a review; Bookman *et al*[20]) and several low molecular weight inhibitors are being developed and/or in clinical studies emphasizing the clinical relevance of HER-2 targeting. In a recent study in different tumor cell lines, synthetic HER-2 siRNAs were shown *in vitro* to reduce HER-2 expression resulting in growth inhibition or apoptosis and upregulation of HLA class 1 expression.[21] In another work, HER-2 silencing upon retrovirus-mediated siRNA transfer led to slower proliferation, increased apoptosis and changes in cell cycle-associated and pro-/anti-angiogenic factors in breast and ovarian cancer cell lines *in vitro*. Upon subcutaneous (s.c.) injection of these *in vitro* pretreated cells, decreased tumor growth was observed.[22]

We generated three synthetic siRNAs against different regions of the HER-2 receptor, which displayed comparable efficiencies and were combined in the subsequent experiments. Treatment of SKOV-3 ovarian carcinoma cells *in vitro* with a single dose of 10 pmol PEI-complexed siRNAs resulted in a ~50% downregulation of HER-2 mRNA already after 24 h. This specific reduction of HER-2 mRNA lasted for at least 72 h and, more importantly, was observed also in the presence of FCS in the culture medium (Figure 2a). Western blotting of cell lysates confirmed the Northern blot results and showed an even more pronounced 65–75% reduction of HER-2 protein levels at days 2 and 3 after treatment (Figure 2b). Concomitantly, p42/44 activation (phosphorylation), which has been shown recently to be reduced upon ribozyme-mediated HER-2 downregulation in SKOV-3 cells,[23] was decreased indicating alterations of molecules downstream in the HER-2 signaling pathways upon siRNA-mediated HER-2 targeting (Figure 2b). The biological relevance of this robust HER-2 downregulation was demonstrated in soft agar assays. In previous studies,[24] colony formation of stably ribozyme-transfected SKOV-3 cells was decreased dependent on the reduction of HER-2 levels. While SKOV-3 cells treated with a PEI-complexed nonspecific control siRNA showed high numbers of large colonies >90 μm, a single treatment with HER-2-specific, PEI-complexed siRNAs led to a ~50% reduction of colony formation (Figure 2c). It is particularly noteworthy that, despite the fact that cells were treated with PEI-complexed siRNAs only once prior to embedding into the agar and were then cultivated for 2–3 weeks without any further treatment, this strong effect on colony formation was observed. The decreased capability of treated cells to form colonies in a soft agar assay is comparable to previous observations in stably HER-2 ribozyme-transfected SKOV-3 cells. While HER-2 depletion also leads to induction of apoptosis, in these studies clonal SKOV-3 cell lines with only ~20% residual HER-2 levels still grew on plastic as well as in soft agar assays.[24] Hence, the reduced colony formation observed here may indicate a combination of a long-lasting intracellular effect, that is, biological half-life of the PEI-delivered siRNAs as well as induction of apoptosis.

In proliferation assays, we furthermore investigated the toxicity of PEI and PEI-complexed siRNAs on SKOV-3 cells. Under standard treatment conditions (10 pmol siRNA/well of a 24-well plate), no toxicity or growth-inhibitory effect of complexed or noncomplexed siRNA, or of the corresponding amount of free PEI, was observed after 2 days (Figure 2d, black bars). The same was true when the cells were treated with three-fold higher amounts (Figure 2d, gray bars) indicating that the dosages used in our experiments were well below any toxic levels. Only a 10-fold excess showed some (PEI/siRNA complex) or considerable (PEI alone) toxicity (Figure 2d, dark gray bars).

Finally, by confocal microscopy we studied the half-life/intracellular fate of fluorescently labeled JetPEI upon transfection of cells with PEI/siRNA complexes. Large and numerous fluorescent dots probably corresponding to endocytotic vesicles were observed in the entire cytoplasm already at early time points (30 or 60 min after addition of the complex to the cells; ie 0 min post-transfection in Figure 2e). When after 1 h the transfection mixture was replaced by normal medium, strong fluorescent signals in the cytoplasm, but never in the nucleus, were still observed for another 30 and 60 min (Figure 2e, middle panels). At 2 h (Figure 2e, right) or 3 h (not shown) post-transfection, however, only very weak fluorescence was detected. Compared to previous studies,[25] we observed a very similar staining pattern while the time-course appears to be faster in our experiments.

Recently, it has been shown that under certain conditions the intratumoral injection of siRNAs displays antitumoral effects.[26] The ultimate goal, however, is the therapeutic use of RNAi through systemic application of a specific RNAi-inducing agent *in vivo*. Therefore, we examined if PEI-complexed siRNAs are stable also under *in vivo* conditions and if intact siRNA molecules reach organs/tissues distant from the site of injection. SKOV-3 cells were subcutaneously injected into the flanks of athymic nude mice and, when tumors reached a size of ~4 × 4 mm, a single dose of ^{32}P-labeled siRNA was injected intraperitoneally. At different time points, the mice were sacrificed and total RNA from various tissues was isolated. As expected, in the case of noncomplexed siRNAs gel electrophoresis of the tissue RNA and subsequent autoradiography of the blotted gel showed no bands indicating the rapid and complete degradation of the radiolabeled siRNA (Figure 3, upper panel, −PEI). In contrast, upon PEI complexation intact siRNA was detected in several tissues already after 30 min as well as after 4 h (Figure 3, +PEI). Since blood was completely negative for ^{32}P-labeled siRNA, the bands represent intact siRNA, which is not located in the residual blood but indeed taken up by the tissue. Notably, compared to other (excretory) organs, particularly strong signals were observed in the tumors probably indicating a preferential uptake due to the EPR (enhanced permeability and retention) effect in addition to high vascularization (see Maeda *et al*[27] for a review). As expected, almost no siRNA was detected in brain indicating that the complex does not cross the blood–brain barrier. The i.p. injection not only proved to be a very simple and reproducible way of application of PEI-complexed siRNAs, but also led to a depot effect of complex residing in the

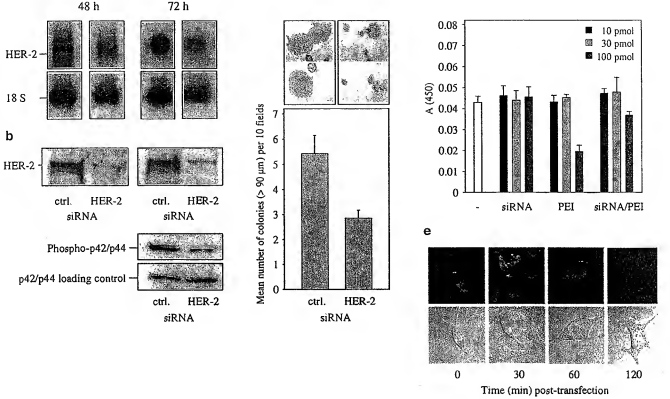

Figure 2 Downregulation of HER-2 (neu/c-erbB-2) in SKOV-3 ovarian carcinoma cells upon a single treatment with PEI-complexed HER-2-specific siRNAs. For HER-2 targeting, three custom-designed siRNAs (CCUGGAACUCACCUACCUGdTdT/CAGGUAGGUGAGUUCCAGGdTdT; CUAC CUUUCUACGGACGUGdTdT/CACGUCCGUAGAAAGGUAGdTdT, GAUCCGGAAGUACACGAUGdTdT/CAUCGUGUACUUCCGGAUCdTdT) were chemically synthesized and annealed (Dharmacon), and the three duplexes were mixed at equimolar ratios. SKOV-3 cells were seeded into six-well plates at 2 × 10⁵ cells/well and, at ~70% confluency, cells were transfected in serum-containing medium by addition of the PEI-siRNA complexes for 48 or 72 h as indicated. Total RNA from cells was isolated using the Tri reagent according to the manufacturer's protocol (Sigma, Taufkirchen, Germany), and 20 µg were separated, blotted, probed for HER-2 as described[33] and, to correct for variability in loading, reprobed with a ^{32}P-labeled 18S cDNA probe. Signals were visualized by autoradiography and quantitated by PhosphoImager analysis. Western blot analysis of equal amounts of cell lysates was performed as described.[23,33] (a) By Northern blotting, a ~50% reduction of HER-2 mRNA was observed after 48 or 72 h, which resulted in a 65–75% decrease of HER-2 protein levels as determined by Western blotting at the same time points (b). Concomitanty, p42/44 activation (phosphorylation) was decreased indicating alterations of molecules downstream in the HER-2 signaling pathways (b, lower panel). (c) In soft agar assays, which were performed as described previously,[34] cells received a single treatment with 0.6 nmol PEI-complexed HER-2 specific or nonspecific siRNA prior to embedding and were allowed to grow for 3 weeks. The reduction of HER-2 levels led to a ~50% decrease of colony formation demonstrating the biological feasibility of HER-2 targeting by PEI complexed siRNAs. (d) SKOV-3 cells were treated with the indicated amounts of complexed or noncomplexed siRNA, or the corresponding amount of free PEI, and after 48 h the numbers of viable cells were determined by WST-1 (Roche). Under standard treatment (10 pmol siRNA/well of a 24-well plate), as well as upon addition of three-fold higher amounts, no toxicity or growth-inhibitory effects were observed (d, black and grey bars) indicating that the dosages used in our experiments were well below any toxic levels. Only a 10-fold excess showed some (PEI/siRNA complex) or considerable (PEI alone) growth inhibition indicating toxicity (dark grey bars). (e) Confocal microscopy of cells transfected with 10 pmol siRNA complexed with fluorescently labeled PEI. Cells were plated on glass coverslips 24 h prior to the transfection with FluoF-PEI/siRNA complexes for 60 min under standard conditions, and were analyzed without fixation at 0–120 min after transfection as indicated in the figure. Excitation of the cells containing FluoF-PEI was achieved at 488 nm and the resulting fluorescence emission was observed using a 505 nm longpass filter. While the nucleus was negative, fluorescent vesicles were observed in the cytoplasm already at early time points and for 1 h post-transfection.

peritoneum for several hours (not shown). Furthermore, since PEIs at higher concentrations tend to show toxicity in the lung, it is important that in our experimental setting siRNA amounts were generally low in the lung, which corresponded well with the absence of microscopically visible changes in lung histology (not shown) and the absence of visible side effects of PEI/siRNA treatment.

Previously, by ribozyme-targeting we have demonstrated that the stable reduction of HER-2 expression in SKOV-3 cells reduces subcutaneous tumor growth in an athymic nude mouse model indicating that HER-2 expression is rate-limiting for tumor growth *in vivo*.[28,29] Therefore, we used this model to test if the systemic application of HER-2-specific, PEI-complexed siRNAs results in the growth inhibition of established s.c. tumors. SKOV-3 cells were subcutaneously injected into the flanks of athymic nude mice, and when tumors reached a size of ~10 mm², animals were treated every 2–3 days with 0.6 nmol PEI-complexed HER-2 siRNAs or a PEI-complexed unrelated siRNA as negative control. As shown in Figure 4A, tumors in mice treated with an unrelated PEI-complexed siRNA grew very well reaching a mean size of >80 mm² after 2 weeks. The treatment with the HER-2-specific siRNAs, however, resulted in a significantly reduced tumor growth. Differences were obvious already after ~1 week of treatment and statistical significance was reached at day 12. Also,

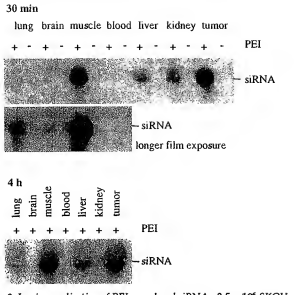

Figure 3 In vivo application of PEI-complexed siRNAs. 2.5 × 10⁶ SKOV-3 ovarian carcinoma cells per site were injected subcutaneously into athymic nude mice (nu/nu), and tumors were allowed to grow until they reached a size of ~4 × 4 mm after 5 days. ³²P-labeled siRNAs, complexed (+) or not complexed (−) with PEI, were injected i.p. into tumor-bearing mice, and after 30 min (upper panels) or 4 h (lower panel) total RNA from various organs and tissue homogenates was prepared as described above and subjected to agarose gel electrophoresis prior to blotting and autoradiography. The bands represent intact ³²P-labeled siRNA, which for several hours is mainly found in tumor and muscle as well as in the liver and, time-dependently, in the kidney. Only little siRNA amounts are detected in the lung and traces in the brain.

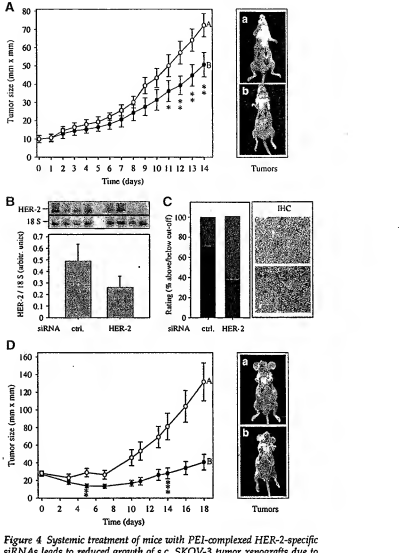

Figure 4 Systemic treatment of mice with PEI-complexed HER-2-specific siRNAs leads to reduced growth of s.c. SKOV-3 tumor xenografts due to decreased HER-2 expression. Subcutaneous tumor xenografts in athymic nude mice were generated as described above. (A) Mice were injected i.p. with 0.6 nmol nonspecific (A, open circles) or HER-2 specific (B, closed circles) PEI-complexed siRNAs 2–3 times per week and tumor sizes were evaluated daily from the product of the perpendicular diameters of the tumors. Differences in tumor growth were visible after ~1 week and reached statistical significance at day 11. Mean ± s.e.m.) is depicted. Student's unpaired t-test was used for comparisons between data sets (< 0.05, ** < 0.03), and examples of tumors (arrows) at day 14 are shown in panel a right. (B) After 2 weeks, tumors were removed, and Northern blotting revealed a ~50% reduction of HER-2 mRNA levels in the treatment group. (C) These findings were confirmed by immunohistochemical analysis of the tumor sections, which was performed as previously described [36] using mouse monoclonal anti-HER2 antibodies, 1:400 (Ab 17, Neomarkers, Fremont, CA, USA). A marked reduction of HER-2 staining intensity and abundance upon treatment with PEI-complexed HER-2-specific siRNAs was observed. (D) i.p. injection of naked HER-2-specific siRNAs fails to exert an inhibitory effect on tumor growth (open circles; compare curves A in A and D) while, again, PEI-complexation of HER-2 siRNA results in a significantly reduced tumor growth. In this experiment, the siRNA/PEI effect was stronger as compared to panel (A) reaching significance already at day 5, which may be due to a slightly earlier onset of the treatment after established tumors were visible. Mean ± s.e.m. is depicted. Student's unpaired t-test was used for comparisons between data sets (** < 0.03, *** < 0.01), and examples of tumors (arrows) at day 18 are shown on the right.*

analysis of the tumors revealed a ~50% reduction of HER-2 mRNA levels in the group treated with the PEI-complexed, HER-2-specific siRNAs (Figure 4B, left), which is consistent with the *in vitro* effects. To determine HER-2 protein levels, tumor sections were immunohistochemically stained and evaluated for HER-2 membrane staining. While the HER-2 staining was heterogeneous in all tumor sections independent of the treatment regimen, the blinded rating of the whole sections for staining intensity (no staining = 0 to very strong staining = 3) and abundance of stained areas revealed that 63% of the tumors of the control group were above the cutoff (defined as the mean of the scores of all sections) while this number dropped to 29% in the treatment group (Figure 4C). To test if HER-2-specific siRNAs exert an inhibitory effect on tumor growth also without prior PEI complexation, the experiment was repeated with i.p. injection of naked compared to PEI-complexed HER-2 siRNA (Figure 4D). The comparison of curves A in Figure 4A and D shows that the injection of naked siRNA resulted in no reduction of tumor growth since in both experiments the tumors show the same growth kinetics reaching a mean tumor size of 70–80 mm² at day 14. Again, PEI-complexed, HER-2-specific siRNAs resulted in a significantly reduced tumor growth (Figure 4D). In this experiment, the siRNA/PEI effect was even stronger, which may be due to a slightly earlier (~2 days) onset of the treatment after established tumors were visible. From these data, we conclude that PEI significantly improves the *in vivo* efficiency of siRNAs.

So far, only very few studies describe the *in vivo* use of siRNAs using either very high siRNA amounts (~4 nmol) injected close to the target organ[30] or showing rather poor tissue penetration and little effects,[31] or relying on the direct injection of the siRNAs into the tumor region.[26] In this paper, we present a novel system providing simultaneously the protection and efficient exogenous delivery of any siRNA *in vivo* upon simple systemic application and without any chemical modification. The effects observed in our *in vivo* tumor model upon targeting of the HER-2 receptor proves that PEI complexation of siRNAs offers an avenue for the development of highly efficient, specific and safe agents for therapeutical applications.

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EXHIBIT 20

nature
chemical biology

RNAi therapeutics: a potential new class of pharmaceutical drugs

David Bumcrot, Muthiah Manoharan, Victor Koteliansky & Dinah W Y Sah

The rapid identification of highly specific and potent drug candidates continues to be a substantial challenge with traditional pharmaceutical approaches. Moreover, many targets have proven to be intractable to traditional small-molecule and protein approaches. Therapeutics based on RNA interference (RNAi) offer a powerful method for rapidly identifying specific and potent inhibitors of disease targets from all molecular classes. Numerous proof-of-concept studies in animal models of human disease demonstrate the broad potential application of RNAi therapeutics. The major challenge for successful drug development is identifying delivery strategies that can be translated to the clinic. With advances in this area and the commencement of multiple clinical trials with RNAi therapeutic candidates, a transformation in modern medicine may soon be realized.

RNAi is a fundamental cellular mechanism for silencing gene expression that can be harnessed for the development of new drugs[1,2]. The reduction in expression of pathological proteins through RNAi is applicable to all classes of molecular targets, including those that are difficult to modulate selectively with traditional pharmaceutical approaches involving small molecules or proteins. Consequently, RNAi therapeutics as a drug class have the potential to exert a transformational effect on modern medicine. In RNAi, the target mRNA is enzymatically cleaved, leading to decreased abundance of the corresponding protein, and specificity is a key feature of the mechanism. Synthetic small interfering RNAs (siRNAs) leverage the naturally occurring RNAi process in a manner that is consistent and predictable with regard to extent and duration of action. In addition, viral delivery of short hairpin RNAs (shRNAs) represents an alternative strategy for harnessing RNAi. Both nonviral delivery of siRNAs and viral delivery of shRNAs are being advanced as potential RNAi-based therapeutic approaches.

In this review, we provide an overview of the molecular mechanism of RNAi; the *in silico* design of siRNAs and shRNAs that are specific for a target of interest, in the context of current concepts relating chemical structure to specificity and potency; the use of chemical modifications that confer stability against exo- and endonucleases present in biological fluids and tissues; and strategies for facilitating cellular delivery *in vivo* through conjugation, complexation and lipid-based approaches to facilitate cellular uptake. We summarize the numerous publications to date demonstrating the robust efficacy of RNAi in animal models of human disease upon direct (local) as well as systemic administration. These proof-of-concept studies support RNAi as the basis for a new therapeutic approach that has the potential to change the treatment of human disease. Most importantly, as we will discuss, clinical trials have recently commenced, with RNAi therapeutic candidates under study for treatment of age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) infection.

Molecular mechanism of RNAi

The RNase Dicer initiates RNAi by cleaving double-stranded RNA substrates into small fragments of about 21–25 nucleotides in length (Fig. 1). These siRNA duplexes are incorporated into a protein complex called the RNA-induced silencing complex (RISC; Fig. 1). Biochemical analysis identified Argonaute 2 (Ago2) as the protein in RISC responsible for mRNA cleavage[3], and the crystal structure of RNA-bound Ago2 has been reported, revealing key interactions[4].

Before RISC activation, the sense (nonguide) strand of the siRNA duplex is cleaved by Ago2, in the same manner as it cleaves mRNA substrates[5,6]. Preventing sense-strand cleavage by chemical modification can reduce siRNA potency *in vitro*; however, experimental context is important, as siRNAs with highly stabilized (uncleavable) sense strands can be highly active.

Role of chemical modifications

Small-molecule pharmaceutical drugs, almost without exception, meet the 'Lipinski Rules', criteria including high lipophilicity and molecular weight of not more than 500. In sharp contrast, siRNAs naturally lack these drug-like properties owing to their large size (two turns of a nucleic acid double helix), nearly 40 anionic charges due to the phosphodiester backbone, and high molecular weight (over 13 kDa). In aqueous solution, with their sugar-phosphate backbone exposed to water, siRNAs are extremely hydrophilic and heavily hydrated. Furthermore, siRNAs are unstable in serum as a result of degradation by serum nucleases, contributing to their short half-lives *in vivo*[7]. Although the molecular weight of siRNAs cannot be reduced, these molecules can be made more 'drug-like' through judicious use of chemical modification to the sugars, backbone or bases of the oligoribonucleotides.

Chemically modified siRNA duplexes have been evaluated in cell-based assays and in animal models. The modifications discussed are

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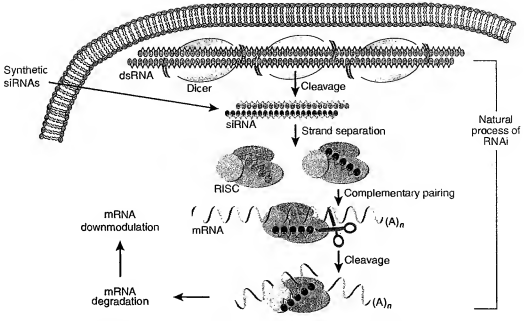


Figure 1 Cellular mechanism of RNA interference. Long double-stranded RNA (dsRNA) is cleaved, by the enzyme Dicer, into small interfering RNA (siRNA). These siRNAs are incorporated into the RNA-induced silencing complex (RISC), where the strands are separated. The RISC containing the guide or antisense strand seeks out and binds to complementary mRNA sequences. These mRNA sequences are then cleaved by Argonaute, the enzyme within the RISC responsible for mRNA degradation, which leads to mRNA down-modulation. A, adenosine.

shown in Figure 2. Stability against nuclease degradation has been achieved by introducing a phosphorothioate (P=S) backbone linkage at the 3′ end for exonuclease resistance and 2′ modifications (2′-OMe, 2′-F and related) for endonuclease resistance[8–10]. An siRNA motif, consisting entirely of 2′-*O*-methyl and 2′-fluoro nucleotides, has enhanced plasma stability and increased *in vitro* potency. At one site, this motif shows >500-fold improvement in potency over the unmodified siRNA[11]. Using phosphatase and tensin homolog (PTEN) as a target, the effect of 2′ sugar modifications such as 2′-fluoro-2′-deoxynucleoside (2′-F), 2′-*O*-methyl (2′-*O*-Me) and 2′-*O*-(2-methoxyethyl) (2′-*O*-MOE) in the guide and nonguide strands was evaluated in HeLa cells. The activity depends on the position of the modification in the guide-strand sequence. The siRNAs with modified residues at the 5′ end of the guide strand seem to be less active than those modified at the 3′ end. The 2′-F sugar is generally well tolerated on the guide strand, whereas the 2′-*O*-MOE modification results in loss of activity regardless of placement position in the construct. The incorporation of 2′-*O*-Me and 2′-*O*-MOE in the nonguide strand of siRNA does not have a notable effect on activity[12]. Sugar modifications such as 2′-*O*-Me, 2′-F and locked nucleic acid (LNA, with a methylene bridge connecting 2′ and 4′ carbons) seem to be able to reduce the immunostimulatory effects of siRNAs (see below).

Duplexes containing the 4′-thioribose modification have a stability 600 times greater than that of natural RNA[13]. Crystal structure studies reveal that 4′-thioriboses adopt conformations very similar to the C3′-endo pucker observed for unmodified sugars in the native duplex[14]. Stretches of 4′-thio-RNA were well tolerated in both the guide and nonguide strands. However, optimization of both the number and the placement of 4′-thioribonucleosides is necessary for maximal potency. These optimized siRNAs are generally equipotent with or superior to native siRNAs and show increased thermal and plasma stability. Furthermore, substantial improvements in siRNA activity and plasma stability have been achieved by judicious combination of 4′-thioribose with 2′-*O*-Me and 2′-*O*-MOE modifications[15].

As mentioned, phosphorothioate (P=S) modifications are generally well tolerated on both strands and provide improved nuclease resistance. The 2′,5′-phosphodiester linkages seem to be tolerated in the nonguide but not the guide strand of the siRNA[16]. In the boranophosphate linkage, a nonbridging phosphodiester oxygen is replaced by an isoelectronic borane (BH_3-) moiety. Boranophosphate siRNAs have been synthesized by enzymatic routes using T7 RNA polymerase and a boranophosphate ribonucleoside triphosphate in the transcription reaction. Boranophosphate siRNAs are more active than native siRNAs if the center of the guide strand is not modified, and they may be at least ten times more nuclease resistant than unmodified siRNAs[17,18].

siRNA duplexes containing the 2,4-difluorotoluyl ribonucleoside (rF) were synthesized to evaluate the effect of noncanonical nucleoside mimetics on RNA interference. Thermal melting analysis showed that the base pair between rF and adenosine is destabilizing relative to a uridine-adenosine pair, although it is slightly less destabilizing than other mismatches. The crystal structure of a duplex containing rF-adenosine pairs shows local structural variations relative to a canonical RNA helix. As the fluorine atoms cannot act as hydrogen bond acceptors and are more hydrophobic than uridine, a well-ordered water structure is not seen around the rF residues in both grooves. Rapid amplification of 5′ complementary DNA ends (5′-RACE) analysis confirms cleavage of target mRNA opposite to the rF placement site[19,20].

Certain terminal conjugates have been reported to improve or direct cellular uptake. For example, siRNAs conjugated with cholesterol improve *in vitro* and *in vivo* cell permeation in liver cells[6]. As described below, cholesterol and an RNA aptamer conjugation show promise in animal models.

Design considerations for potency and specificity

Critical design concerns in the selection of siRNA duplexes for therapeutic use are potency and specificity. There are two major considerations

with regard to siRNA specificity: 'off-targeting' due to silencing of genes sharing partial homology with the siRNA, and 'immune stimulation' due to the engagement of components of the innate immune system by the siRNA duplex. A combination of bioinformatics methods, chemical modification strategies and empirical testing is required to address these issues.

Concomitant with the first description of the structure of active siRNAs, a set of 'rules' was proposed for selecting potent siRNA duplex sequences[21,22]. Several groups have subsequently developed more sophisticated extensions of these largely empirical criteria, leading to the development of algorithms for siRNA design[23,24]. Recent biochemical studies of the molecular mechanism of RNA interference have highlighted some key features of potent siRNA duplexes (Fig. 3). Most notably, it has been found that the efficiency with which the guide strand is incorporated into the RISC complex is perhaps the most important factor determining siRNA potency. Because siRNA duplexes are symmetric, the question arose of how the RISC machinery is able to determine which strand to use for target silencing. Insight into this enigma came from careful analyses of microRNAs (miRNAs), the endogenous counterparts of siRNAs. Examination of the sequences of a large number of vertebrate and invertebrate miRNA precursor sequences showed that the predicted thermodynamic stabilities of the two ends of the duplex are unequal[25,26]. Specifically, calculating the ΔG for the several base pairs at each end of the duplex revealed that the 5′ end of the mature miRNA pairs less tightly with the carrier strand than does the 3′ end. In short, miRNA precursors show thermodynamic asymmetry. It was hypothesized that components of the RISC machinery select the guide strand based on this asymmetry.

Experimental evidence supporting the asymmetry hypothesis has been derived from studies using chemically synthesized siRNAs in transfection experiments. Through an elegant assay in which each strand of the siRNA targets a different reporter gene, Schwarz *et al.* were able to quantify the relative efficiency of RISC incorporation for each of the two strands[25]. They found that the RISC machinery preferentially incorporates the strand whose 5′ end binds less tightly with the other strand. In fact, strand selection could be switched by making a single nucleotide substitution at the end of the duplex to alter relative binding of the ends. A similar conclusion was reached by another group based on *in vitro* screening of a large collection of siRNAs with varying potency[26,27]. Thus, designing siRNAs with relatively weaker base pairing at the 5′ end of the desired guide strand may increase the likelihood of obtaining a potent duplex.

The issue of off-target silencing has been the subject of intensive study in a number of different laboratories over the past several years. Transcriptional profiling studies have confirmed that siRNA duplexes can potentially silence multiple genes in addition to the intended target. As expected, genes in these so called off-target 'signatures' contain regions that are complementary to one of the two strands in the siRNA duplex[28–30]. More detailed bioinformatic analyses have revealed that the regions of complementarity are most often found in the 3′ UTRs of the off-target genes[31]. This immediately suggested a microRNA-like mechanism, because miRNAs generally interact with the 3′ UTR region of their targets. Evidence in support of this concept came from a closer look at the determinants of siRNA off-targeting. It was discovered that sequence complementarity between the 5′ end of the guide strand and the mRNA is the key to off-target silencing[31,32]. The critical nucleo-

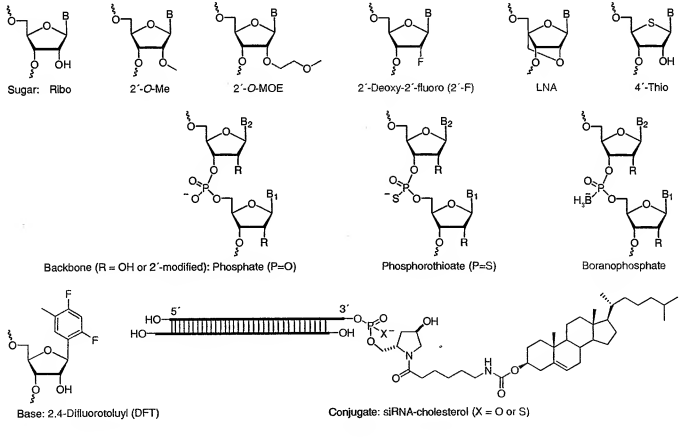


Figure 2 Chemical modifications of siRNAs. Shown are structures of sugar, backbone and base modifications and of the cholesterol conjugate.

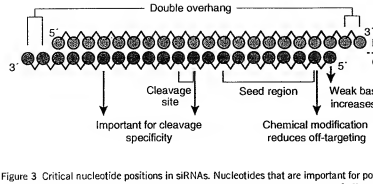


Figure 3 Critical nucleotide positions in siRNAs. Nucleotides that are important for potency, mRNA recognition, mRNA cleavage and cleavage specificity, including minimization of off-targeting, are shown.

tides were found to be positions 2–8, counting from the 5′ end of the guide strand (Fig. 3). This corresponds to the so-called 'seed region' of miRNAs, which has been shown to determine miRNA specificity[33].

Two strategies for avoiding seed region–mediated off-targeting can be envisioned. The first is simply to ensure that nucleotides complementary to positions 2–8 of the guide strand are unique to the intended target. Though theoretically possible, this approach may prove impractical, as the universe of possible seed-region heptamers is only 16,384 distinct sequences. Even if the homology is restricted to the 3′ UTR, it may prove difficult to identify siRNA duplexes satisfying the criteria of potency and specificity. As one alternative, recent published work has reported that off-targeting can be substantially reduced by chemical modification of nucleotides within the seed region[34]. Specifically, the introduction of a 2′-*O*-*Me* modification into nucleotides within the seed region was shown to inactivate the off-target activity of the siRNA without compromising silencing of the intended mRNA. In fact, introduction of the modification at a single nucleotide position (position 2, Fig. 3) is sufficient to suppress the majority of off-targeting. The mechanism, anticipated by recently published crystal structure data, appears to involve perturbation of RISC interaction with the modified nucleotide.

Interactions outside of the seed region can also substantially affect siRNA specificity. Although the seed region seems to be critical for mRNA recognition, notable mRNA cleavage requires more extensive base pairing between the siRNA and the target[32]. In a recent study, Schwarz *et al.* designed siRNAs capable of distinguishing between mRNA targets that differ by only one nucleotide[35]. They showed that target selectivity depends on the location of the mismatch between the siRNA and the mRNA. Whereas positioning the mismatch within the seed region imparts a certain degree of selectivity, positioning the mismatch further 3′ in the guide strand (especially at positions 10 and 16, Fig. 3) produces highly discriminatory siRNAs. The authors hypothesized that mismatches at these positions are particularly disruptive to the helical structure of the siRNA–mRNA complex required for target cleavage.

A second mechanism whereby siRNA duplexes can induce unintended effects is through stimulation of the innate immune system in certain specialized immune cell types. It has been demonstrated that siRNA duplexes harboring distinct sequence motifs can engage Toll-like receptors (TLRs) in plasmacytoid dendritic cells, resulting in increased production of interferon[36]. Such immune stimulation could pose a significant problem in a therapeutic setting. This phenomenon is reminiscent of the results of earlier studies with DNA antisense oligonucleotides in which distinct sequences (so-called CpG motifs) were shown to be immunostimulatory[37]. Subsequent studies established that TLR-9, the receptor for unmethylated CpG-containing pathogen DNA, is the innate immune regulator engaged by antisense oligonucleotides[38]. In the case of siRNAs, it seems to be TLR-7 that is the mediator of immune stimulation[36].

Several possible strategies exist for avoiding immune stimulation by siRNA duplexes, including avoidance of the offending sequences during siRNA design and chemical modification to inactivate the motifs. The former approach is not feasible at present because the full spectrum of stimulatory motifs has not been identified. Evidence supporting the latter approach comes from studies in which chemical modifications at the 2′ position of nucleotides within putative TLR-7–interacting sequences eliminate immune stimulation without compromising silencing activity[36,39]. Another possibility would be to use siRNA delivery strategies that avoid the cell types responsible for immune stimulation.

Prediction of the nucleotide sequence and chemical modifications required to yield an ideal siRNA duplex remains a work in progress. Still, the recent advances described above have allowed the development of design algorithms that greatly increase the likelihood of success. It is nonetheless important to note that the relevance of *in vitro* measurements of potency and specificity to *in vivo* activity in a therapeutic setting has yet to be established. For example, the spectrum of off-target genes identified in tissue culture studies can differ depending on the method by which siRNAs are introduced into cells[40]. Also, the induction of an innate immune response by certain siRNA sequences is cell type dependent[41]. At present, the most prudent and robust strategy is to synthesize and screen a substantial library of siRNA duplexes for each target of interest (perhaps even 'tiling' the entire messenger RNA) to identify the most promising candidates.

Proof of concept for local RNAi in animal models

During the past several years, numerous studies have been published demonstrating efficacious silencing of disease genes by local administration of siRNAs or shRNAs in animal models of human disease. Both exogenous and endogenous genes have been silenced, and promising *in vivo* results have been obtained across multiple organs and tissues. Efficacy has been demonstrated for viral infection (respiratory and vaginal), ocular disease, disorders of the nervous system, cancer and inflammatory bowel disease (Fig. 4). An important aspect of these proof-of-concept studies is that they have supported the expected high specificity of RNAi.

Local RNAi can protect against both respiratory[42,9] and vaginal[43] viral infections. Two reports illustrate efficacious direct delivery of siRNA to the lung in rodent and monkey models of RSV, influenza and severe acute respiratory syndrome (SARS) infection with and without lipid formulation. In mouse models of infection, pulmonary viral titers of RSV and parainfluenza were reduced by more than 99% with intranasal delivery of siRNAs formulated with TransIT-TKO, a cationic polymer–based transfection reagent, targeting RSV and parainfluenza virus, respectively[42]. In addition, siRNA targeting RSV reduced pulmonary pathology, as assessed by respiratory rate, leukotriene induction and inflammation. These positive proof-of-concept studies in mice have led to clinical trials of RNAi therapeutics targeting RSV.

Another system for which there have been multiple examples of efficacious local delivery of siRNA is the eye, where proof of concept has been successfully achieved in animal models of ocular neovascularization and scarring using saline and lipid formulations[44–46]. Intravitreal injection

of siRNA targeting vascular endothelial growth factor (VEGF) receptor-1, formulated in phosphate-buffered saline, was effective in reducing the area of ocular neovascularization by one-third to two-thirds in two mouse models[44]. In addition, siRNAs targeting VEGF and the transforming growth factor-β receptor type II, formulated with TransIT-TKO, were injected directly into the mouse eye, resulting in inhibition of laser photocoagulation–induced choroidal neovascularization[45] and latex bead–induced collagen deposition and inflammatory cell infiltration[46], respectively. As with the lung, multiple siRNA formulations were effective in the eye. These encouraging proof-of-concept studies in animal models have led to clinical trials of siRNAs targeting the VEGF pathway in AMD.

In the nervous system, RNAi has been particularly useful for validating disease targets *in vivo*. Again, several formulations, including saline, polymer complexation and lipid or liposomal formulations, have been efficacious for delivering siRNAs locally to the nervous system in numerous disease models. The simplest mode of delivery is intracerebroventricular, intrathecal or intraparenchymal infusion of naked siRNA formulated in buffered isotonic saline, which results in silencing of specific neuronal molecular mRNA targets in multiple regions of the central and peripheral nervous systems[47–50]. With naked siRNA formulated in buffered isotonic saline, doses of 0.4 mg per day are typically required for effective target gene silencing. Polymer complexation and lipid or liposomal formulations such as polyethylene imine (PEI), iFECT, DOTAP and JetSI/DOPE facilitate cellular uptake and reduce the doses of siRNA required for effective neuronal target silencing *in vivo* to approximately 5–40 μg[51–54].

Local viral delivery of shRNA to the nervous system has been reported *in vivo* with adenoviral, adeno-associated viral (AAV) and lentiviral delivery in normal mice[55] as well as in animal models of spinocerebellar ataxia[56], Huntington disease[57,58], amyotrophic lateral sclerosis (ALS)[59,60] and Alzheimer disease[61], where abnormal, disease phenotypes including behavior and neuropathology were normalized. Notably, all of the *in vivo* studies to date have targeted genes expressed in neurons; it remains to be seen whether silencing *in vivo* can be achieved in other nervous-system cell types such as oligodendrocytes and astrocytes. Moreover, for endogenous neuronal targets, expression of the target gene is typically reduced only partially, and in some cases by as little as 10–20%, yet this modest reduction in mRNA results in a marked effect on the specific behavior appropriate to the targeted gene.

For application to oncology, direct delivery of siRNAs and viral delivery of shRNAs to tumors have been successful in inhibiting xenograft growth in several mouse models. A number of approaches—including lipid-based formulation (TransMessenger[62]) and complexation with PEI[63], cholesterol-oligoarginine[64], a protamine-Fab fusion protein[65] and atelocollagen[66,67]—have been shown to facilitate delivery into tumor cells. Notably, these siRNA delivery approaches are effective with several or even a single intratumor injection of siRNA, at microgram doses. Very recently, aptamer-siRNA chimeric RNAs have also been used successfully to facilitate siRNA delivery *in vivo*, resulting in tumor regression in a xenograft model of prostate cancer[68]. Viral and vector-based delivery of shRNAs directly to the tumor site[69,70] has also been used effectively in mouse models of adenocarcinoma, Ewing sarcoma and prostate cancer. Of the multiple delivery strategies that have been effective in mouse tumor models, the aptamer approach has the potential of substantially simplifying delivery; if an aptamer is available for a tumor-specific receptor such as prostate-specific membrane antigen (PMSA) and the large-scale synthesis of such a construct is feasible.

For inflammatory bowel disease, direct delivery of siRNA targeting tumor necrosis factor-α (TNF-α) with a Lipofectamine formulation has recently been shown to reduce not only TNF-α abundance but also colonic inflammation after administration by enema[71]. This report, together with a study of siRNA targeting herpes simplex virus-2 (ref. 43), suggests that mucosal surfaces are accessible with liposomal siRNA formulations.

Proof of concept for systemic RNAi in animal models

Over the past several years, a number of studies have been published demonstrating the silencing of disease genes by systemic administration of siRNAs (Fig. 4; reviewed in refs. 1,72). In some of these studies, silencing of endogenously expressed genes has shown promising *in vivo* results in different disease contexts. For example, efficacy has been demonstrated in mouse models of hypercholesterolemia and rheumatoid arthritis. In other work, systemic RNAi targeting exogenous genes has shown promise in models of viral infection (hepatitis B virus (HBV), influenza virus, Ebola virus) and in tumor xenografts. Critical to the success of most of these studies has been the use of chemical modifications or delivery formulations that impart desirable pharmacokinetic properties to the siRNA duplex and that also promote cellular uptake in tissues.

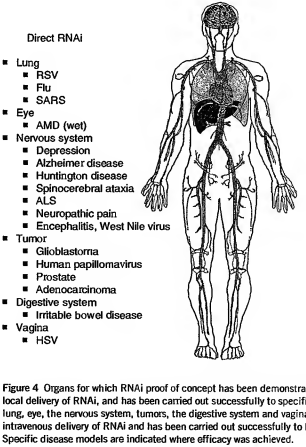


Figure 4 Organs for which RNAi proof of concept has been demonstrated. Direct RNAi represents local delivery of RNAi, and has been carried out successfully to specific tissues or organs, including lung, eye, the nervous system, tumors, the digestive system and vagina. Systemic RNAi represents intravenous delivery of RNAi and has been carried out successfully to lung, tumors, liver and joint. Specific disease models are indicated where efficacy was achieved.

In 2004, Soutschek *et al.* demonstrated effective silencing of the apolipoprotein apoB in mice by intravenous administration of cholesterol-conjugated siRNA duplexes[6]. Three daily injections of cholesterol-conjugated siRNA at a dose of 50 mg kg^{-1} resulted in silencing of the apoB mRNA by 57% and 73%, respectively, in the liver and jejunum, the two principal sites for apoB expression. The mechanism of action was proven, by 5′-RACE, to be RNAi mediated. This mRNA silencing produced a 68% reduction in apoB protein abundance in plasma and a 37% reduction in total cholesterol. These therapeutically relevant findings were completely consistent with the known function of apoB in lipid metabolism. Cholesterol conjugation imparted critical pharmacokinetic and cellular uptake properties to the siRNA duplex.

Further advances in systemic RNAi with optimized delivery have recently been reported. Recently, Zimmermann *et al.* made use of siRNA duplexes formulated in stable nucleic acid lipid particles (SNALPs[39]) to recapitulate the silencing of apoB in mice[73]. In rodent studies, the silencing produced by a single dose of SNALP-formulated siRNA at 2.5 mg kg^{-1} was greater than that reported in the earlier study using cholesterol-conjugated siRNAs. More importantly, therapeutic silencing of apoB was also demonstrated in nonhuman primates. A single dose of 2.5 mg kg^{-1} siRNA encapsulated in the SNALP formulation reduced apoB mRNA in the livers of cynomolgus monkeys by more than 90%. As in the mouse experiments, apoB silencing was accompanied by substantial reductions in serum cholesterol (>65%) and low-density lipoproteins (>85%). Furthermore, silencing was shown to last for at least 11 d after a single dose. In addition, the treatment seemed to be well tolerated, with transient increases in liver enzymes as the only reported evidence of toxicity. This primate study represented an important step forward in the development of systemic RNAi for therapeutic applications. Moreover, the general applicability of SNALP formulations for hepatic delivery of siRNA has been demonstrated in animal models of HBV and Ebola virus infection[74,75].

In mouse tumor xenograft models, the efficacy of systemic RNAi has been demonstrated using a variety of delivery strategies (reviewed in refs. 1,2,76). Systemically delivered cationic cardiolipin liposomes containing siRNA specific for Raf-1 inhibit tumor growth in a xenograft model of human prostate cancer[77]. Vascular endothelial growth factor receptor-2 (VEGF-R2)-targeting siRNAs complexed with self-assembling nanoparticles consisting of polyethylene glycol-conjugated (PEGylated) PEI with an Arg-Gly-Asp peptide attached at the distal end of the PEG accumulate in tumors and cause inhibition of VEGF-R2 expression. Intravenous administration of these complexes into tumor-bearing mice inhibits both tumor angiogenesis and growth rate[78]. Simpler PEI formulations have also shown efficacy in xenograft tumor models[79], as have complexes of siRNA duplexes with atelocollagen. Systemic administration of atelocollagen–siRNA complexes has marked effects on subcutaneous tumor xenografts[66] as well as bone metastases[76]. Another recently described delivery strategy made use of a recombinant antibody fusion protein to achieve cell type–specific delivery. As described above, Song *et al.* fused the nucleic acid binding protein protamine to the C terminus of a fragment antibody (Fab) targeting the HIV-1 envelope protein gp160. After systemic administration, the Fab-protamine fusion was able to deliver an siRNA mixture to mouse melanoma cells engineered to express the envelope protein, leading to substantial inhibition of tumor growth in mice. Tumors derived from cells not expressing the envelope protein were unaffected. In another example of ligand-directed delivery, Hu-Lieskovan *et al.* made use of transferrin-conjugated nanoparticles to deliver an siRNA targeting the oncogenic EWS-FLI translocation-derived mRNA in a mouse model of metastatic Ewing sarcoma[80]. Removal of the targeting ligand or the use of a control siRNA sequence eliminated the antitumor effects.

Comparison of different delivery strategies for RNAi

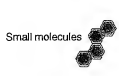
Effective delivery is perhaps the most challenging remaining consideration for successful translation of RNAi to the clinic and to broad use in patients. In the animal studies reviewed above, nonviral and viral approaches, local and systemic administration, and multiple formulations (saline, lipids, and complexes or conjugates with small molecules, polymers, proteins and antibodies) have all been used to achieve efficacy. However, each of these approaches has distinct advantages and disadvantages for clinical translation, which require careful consideration.

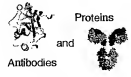
Although viral delivery provides the potential advantage that a single administration could lead to durable down-modulation of the targeted pathological protein, a major risk was highlighted recently[81]. With AAV delivery of shRNAs, excessive diversion of the endogenous RNAi mechanism occurred that resulted in pronounced toxicity in mice. Clearly, for all drugs, it is critical to be able to control the level of drug and the duration of drug action, such that the exposure is safe while still being efficacious. In distinct contrast to nonviral delivery of siRNAs, a substantial liability of viral delivery is that it is impossible to fully predict drug exposure, with regard to both amount and timing. In addition, as shRNAs enter the RNAi pathway upstream of therapeutic applications for siRNAs, viral vectors expressing high levels of shRNAs may interfere with endogenous miRNA biogenesis.

The principal considerations for selecting local versus systemic siRNA administration are the doses needed to achieve sufficient drug concentration in the target tissue and the possible effects of the exposure of nontargeted tissues to drug. At one extreme, with certain tissues, efficacy has so far been demonstrated only with local delivery; current formulations may not provide sufficient drug concentration in the target tissue after systemic delivery. However, with other tissues (for example, liver), intravenous doses in the low mg kg^{-1} range with liposomal formulation provide robust therapeutic gene silencing. In general, and as with any pharmacologic approach, the doses of siRNA required for efficacy are substantially lower when siRNAs are injected into or near the target tissue than when they are administered systemically. Given the high specificity of siRNAs for their intended molecular target, exposure of nontargeted tissues to drug is an issue only if the molecular target is expressed in nontargeted tissue and has an important role in normal cellular function within that tissue. In these cases, local delivery with more focused exposure might circumvent undesired side effects resulting from systemic delivery.

Liposomes, and lipid complexes or conjugates with small molecules, polymers, proteins and antibodies, have all been used to facilitate delivery of siRNAs to target cells. With these delivery partners, more robust efficacy can be achieved with doses of siRNA that are substantially lower, less frequent or both. For the additional (non-siRNA) components, however, there are associated biological and large-scale manufacturing considerations. Lipids and polymers can have cytotoxic effects that might limit their use in siRNA delivery for particular disease indications and dosing paradigms. However, it seems to be possible to identify lipid-based formulations and dosing regimens for which cytotoxicity is minimal and the risk of histopathology is reduced[51–53]. Small molecules, proteins and antibodies used as conjugates also need to be considered from the standpoint of biological activity. If the endogenous molecule (for example, receptor) with which they interact has an important role in normal physiology, then using this endogenous molecule to potentiate delivery may alter its normal function and produce undesired side effects. In all of these cases, the additional non-siRNA molecule or molecules increase the complexity of manufacturing, particularly at large scale. With scientific and technical advances, these approaches may provide marked enhancements to siRNA delivery with acceptable biological and manufacturing considerations.

Table 1 Therapeutic modalities: pros and cons





■ Antagonism or agonism of target	■ Antagonism or agonism of target	■ Antagonism only
■ Extracellular and intracellular targets	■ Extracellular targets	■ All targets, including 'nondruggable targets'
■ Not all target classes can be modulated selectively and potently	■ Highly selective and potent	■ Highly selective and potent
■ Lead ID and optimization slow	■ Lead ID and optimization slow	■ Rapid lead ID and optimization
■ Easy to synthesize	■ Difficult to produce	■ Easy to synthesize

Key features of the two major classes of traditional pharmaceutical drugs—small molecules, and proteins and antibodies—are shown for comparison with RNAi as a therapeutic approach.

Clinical trials with RNAi therapeutics

The first clinical trials with RNAi therapeutics target the VEGF pathway for the wet form of AMD and the RSV genome for treatment of RSV infection; in both cases the initial approach is direct administration of the RNAi therapeutic in a saline formulation. In both these cases, highly validated disease targets are being inhibited with siRNAs. Furthermore, direct administration of siRNAs to the eye and lung for AMD and RSV infection, respectively, maximizes the chances of delivering sufficient and therapeutically relevant concentrations of drug to the tissue of interest. In a phase 2 trial in patients with serious progressive AMD, the siRNA Cand5, targeting VEGF, has been reported to provide dose-related benefits with respect to several endpoints, including near vision and lesion size (http://www.acuitypharma.com/press/release13.pdf). Cand5 is also being tested for efficacy against diabetic macular edema in a phase 2 trial that began in early 2006 (http://www.acuitypharma.com/press/release10.pdf). The siRNA Sirna-027, targeting VEGF receptor-1, has recently completed phase 1 trials in patients with the wet form of AMD, in whom it was reported to be well tolerated. In addition, it was reported to stabilize or improve visual acuity in a subset of patients (http://www.sirna.com/wt/page/ocular). For RSV infection, two phase 1 trials with the siRNA ALN-RSV01 have been completed in over 100 healthy adult volunteers, in one of the largest human studies with an RNAi therapeutic, and ALN-RSV01 was found to be safe and well tolerated (http://phx.corporate-ir.net/phoenix.zhtml?c=148005&p=irol-newsArticle2&ID=849576&highlight=). Additional RNAi therapeutic candidates that are expected to advance into the clinic within the coming year include siRNAs targeting pandemic influenza (http://www.alnylam.com/therapeutic-programs/programs.asp) and hepatitis C (Sirna-034; http://www.sirna.com/wt/page/anti_viral). As these and other trials advance through the clinic in the near future, the exciting potential of siRNAs may be demonstrated.

Comparison of RNAi with traditional pharmaceutical drugs

As a therapeutic approach, RNAi provides solutions to the major drawbacks of traditional pharmaceutical drugs (Table 1). The principal advantages of RNAi over small-molecule and protein therapeutics are that all targets, including 'non-druggable' targets, can be inhibited with RNAi and that lead compounds can be rapidly identified and optimized. The primary challenge associated with small-molecule drugs is the identification of highly selective and potent compounds—a difficult and time-consuming process that, for some targets, can be unsuccessful. With RNAi, the identification of highly selective and potent sequences is rapid and has been demonstrated with numerous molecular targets across all molecular classes. With protein and antibody drugs, the main technical challenge is production. For proteins, acceptable cellular production levels are often difficult to achieve. For biologics as a therapeutic class, aggregation continues to be a major issue. In contrast, siRNAs are synthetic and easy to produce from a chemistry standpoint. Of course, with RNAi, by definition, only antagonism of the specific molecular target is possible, whereas small molecules, proteins and antibodies provide an opportunity for agonism of a molecular target. Overall, however, RNAi holds great promise as a therapeutic approach providing a major new class of drugs that will fill a significant gap in modern medicine.

Conclusions

Significant progress has been made in advancing RNAi therapeutics in a remarkably short period of time. Starting from the discovery that RNAi is mediated by long double-stranded RNA in *Caenorhabditis elegans* by Fire and Mello in 1998[82] and the publication in 2001 by Tuschl and his Max Planck Institute colleagues that synthetic siRNAs can silence target genes in mammalian systems[20], the relatively short years since have seen an explosion in reports on therapeutic applications that harness RNAi. Clearly, the principal challenge that remains in achieving the broadest application of RNAi therapeutics is the hurdle of delivery. That said, tremendous progress has been made with new conjugation, complexation and lipid-based approaches, although the challenge of siRNA delivery has yet to be solved for all cell types in all organs. Once that challenge is met, the development of RNAi therapeutics will be limited primarily by target validation. It will then be possible to rapidly advance RNAi therapeutics against potentially any disease target in clinical studies and to thereby treat disease in a new manner. In the near future, the ongoing clinical trials with siRNAs for macular degeneration and RSV may reveal the exciting potential of RNAi therapeutics as the next major class of drug molecules.

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EXHIBIT 21

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The silent treatment: siRNAs as small molecule drugs

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As soon as RNA interference (RNAi) was found to work in mammalian cells, research quickly focused on harnessing this powerful endogenous and specific mechanism of gene silencing for human therapy. RNAi uses small RNAs, less than 30 nucleotides in length, to suppress expression of genes with complementary sequences. Two strategies can introduce small RNAs into the cytoplasm of cells, where they are active – a drug approach where double-stranded RNAs are administered in complexes designed for intracellular delivery and a gene therapy approach to express precursor RNAs from viral vectors. Phase I clinical studies have already begun to test the therapeutic potential of small RNA drugs that silence disease-related genes by RNAi. This review will discuss progress in developing and testing small RNAi-based drugs and potential obstacles.
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Keywords: *RNA interference; small interfering RNA; therapy; drug development*

Introduction

RNA interference (RNAi) is a ubiquitous mechanism in eukaryotic cells to suppress the expression of genes that determine fundamental cell fate decisions of differentiation and survival.[1,2] In plants and lower organisms RNAi also protects the genome from viruses and insertion of rogue genetic elements, like transposons.[3–6] In RNAi, small double-stranded RNAs processed from long double-stranded RNAs or from transcripts that form stem-loops, silence gene expression by several mechanisms – by targeting mRNA for degradation, by preventing mRNA translation or by establishing regions of silenced chromatin.[7,8] All of these mechanisms suppress expression of genes bearing complementary sequences of at least seven nucleotides.[9–13] mRNA degradation is probably the most powerful mechanism for silencing gene expression and is the most specific. It is this RNAi pathway that therapeutic strategies have been designed to activate.[8] mRNA degradation occurs when one strand (the antisense or guide strand) of the short interfering RNA (siRNA, ~22 nucleotides in length) directs the RNA-induced silencing complex (RISC) that contains the RNA endonuclease Ago2 to cleave its target mRNA bearing a complementary sequence.[14–16] Although some RNAi mechanisms, especially inhibition of translation, do not require extensive base-pairing over the length of the siRNA, efficient mRNA cleavage likely requires high complementarity, thereby providing specificity.[17]

As every cell contains the RNAi machinery and any gene can be targeted with a good deal of (but still imperfect, see below) specificity, the prospect of specifically suppressing the expression of disease-causing genes has generated a lot of enthusiasm for developing RNAi-based therapies. Because RNAi is an endogenous and ubiquitous pathway, the effectiveness of gene silencing achieved with RNAi surpasses what has been possible in the past using other small nucleic acids, such as antisense oligonucleotides or ribozymes.[7,8] In one head-to-head comparison, siRNAs knocked down gene expression about 100–1000 fold more efficiently than antisense oligonucleotides.[18] The high efficiency may be related to the catalytic nature of RNAi, where one siRNA can be used over and over to guide the cleavage of many mRNAs.[19] The high efficiency may also be due to protection of the active component of the siRNA (the antisense or guide strand) from digestion by endogenous RNases by its incorporation into the RISC, although this has not been explicitly demonstrated.

Silencing specificity and potential toxicity from off-target effects

Specificity is such that by clever siRNA design, disease-linked alleles of genes that differ by a single nucleotide polymorphism from their wild-type allele can be targeted for silencing without suppressing the expression of the corresponding wild-type gene.[20–22] Although initial reports suggested that siRNAs would have previously unheard of specificity for their targets, several mechanisms have since been described that can lead to unintended off-target effects on gene expression and need to be seriously considered in developing RNAi-based drugs. One potential problem is induction of the interferon response, which causes global, nonspecific inhibition of protein translation. Although small double-stranded RNAs, less than 30 nucleotides in length, do not efficiently activate a full interferon response,[17] a subset of interferon genes can be activated by siRNAs, particularly in specialized highly sensitive cell lines and at high concentrations of siRNAs.[23–26] Fortunately, this problem

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has not been observed in most animal studies, even when looked at with highly sensitive RT-PCR assays. siRNAs containing GU-rich sequences can also indirectly induce IFN production by binding to Toll-like receptors (TLR3, TLR7 and TLR8) that alarm immune activating cells to the presence of RNA viral pathogens.[27–30] Toxicity from the ensuing inflammatory response, which activates interferons and other proinflammatory cytokine genes, might also pose a therapeutic problem.[23–25] However, binding of double-stranded RNAs to TLRs appears to be sequence specific and may be abrogated by chemical modifications of the sugar backbone of the siRNAs without jeopardizing efficacy.[31] Also the inflammatory effect requires much higher RNA concentrations than those required for gene silencing.

Perhaps more serious are unanticipated off-target effects that occur by siRNA recognition of other mRNAs bearing only partial homology.[32,33] Although microarray studies have shown that differences in off-target mRNA levels are generally small (usually much less than twofold, except for a few genes), effects on protein expression, which is more difficult to survey, might become important if the microRNA pathway of translation inhibition is activated.[32–34] siRNAs can act like microRNAs to inhibit translation, while microRNAs with high homology can direct mRNA degradation.[35,36] The rules for identifying microRNA targets are still not defined well enough to reliably predict genes that might be inadvertently silenced by any particular siRNA. Because microRNA silencing may be activated by base-pairing of only seven consecutive nucleotides, careful comparison of candidate siRNA guide strand sequences with mRNAs in the human genome is a prudent step to diminish potential off-target effects. Although short stretches of homology are unavoidable, longer stretches, which are likely to have a more profound effect on gene expression, can be avoided. These nonspecific off-target effects are not limited to the guide strand, but can also be generated by the sense strand of the double-stranded siRNA if it becomes incorporated into RISC and binds to another set of mRNAs bearing partially complementary sequences.[33] Because RISC incorporation favors the strand whose 5′-end is least thermodynamically stable to unwinding, it is now possible to design siRNAs destabilized at the 5′-end of the guide strand to promote incorporation of only the guide strand and thus minimize this potential problem.[37–39] A recent comparison of the effects that siRNAs and delivery agents had on the nonspecific silencing of gene expression found that the majority of off-target effects were due to the lipid-based transfection reagent and not the siRNA.[40] This is consistent with findings that naked siRNAs produced no detectable interferon response upon injection into mice.[41] Whether off-target silencing of unintended genes will pose a real problem for clinical use remains to be seen. As the microRNA effect on gene expression is weak and is likely to require the concerted action of multiple microRNAs working on a single mRNA to block translation, it may not turn out to be a significant problem in practice.[34]

Another possible source of unintended toxicity might arise if the introduced RNAs compete with limited amounts of Dicer and RISC to interfere with endogenous RNAi pathways required to maintain the cell in its differentiated state. In fact, there is evidence that the RNAi machinery can be limiting.[42] One of the ways adenovirus circumvents a potential host RNAi antiviral effect is to express high amounts of a non-coding RNA stem-loop that interferes with nuclear-cytoplasmic transport by binding to exportin 5 and inhibits processing of host cell siRNA and microRNA precursors by binding to Dicer.[43] Although siRNAs silence at such low concentrations that this source of toxicity is unlikely in most cells, it may set a limit on how many different siRNAs can be used simultaneously in one target cell. In clinical situations, where escape from RNAi-based drugs is likely and where silencing multiple gene targets is therefore a good idea (such as in suppressing viral infection or treating tumors) competition for the RNAi machinery may need to be considered.

Optimizing siRNA design for silencing

Silencing can vary from less than twofold suppression by inefficient siRNAs to undetectable target mRNA even by RT-PCR by some 'super' siRNAs.[44,45] How much a gene is transcribed does not seem to be an important determinant of the extent of silencing as abundant mRNAs appear to be as well silenced as rare mRNAs. Situations where transcription is being activated *de novo* may be particularly fortuitous for therapeutic intervention. To use siRNAs or their small RNA precursors as drugs, they must be efficiently delivered into the cytoplasm of cells, the site of precursor RNA processing by the enzyme Dicer and uptake of the siRNA guide strand into RISC.[19] Because Dicer may direct endogenously processed siRNAs and microRNAs to the RISC, small double-stranded RNAs that are between 25 and 30 nucleotides in length and require Dicer processing appear to be more efficient at inducing RNAi than smaller siRNAs designed for direct incorporation into RISC.[46] Once within the RISC, the durability and efficiency of silencing depend on many factors, most of which are poorly understood, but may include sequence preferences for RISC binding along the siRNA, accessibility of the target site on the mRNA,[47,48] and thermodynamic considerations.[39] Although existing algorithms readily available on several websites (http://www.dharmacon.com; http://www.ambion.com/techlib/misc/siRNA_finder.html; http://molecula.com/new/siRNA_inquiry.html; http://www1.qiagen.com/siRNA) are fairly good at predicting sequences that will work, only experimental testing can identify the most efficient sequences. The most important factor that determines durability of silencing in cells appears to be the rate of cell division, which leads to dilution of the activated RISC as it is divided between daughter cells.[7,8] In rapidly dividing tumor cells, used for most of the early RNAi *in vitro* experiments, silencing peaks 2–3 days after transfecting siRNAs and begins to wane around day 5 and disappears by day 7.[49] However, in terminally differentiated non-dividing cells, such as macrophages, silencing lasts for as long as the cells can be cultured (several weeks).[44] There is also some suggestion that the persistence of the activated RISC for any siRNA may depend on the presence of the target mRNA.[44] For therapeutic purposes, the rate of division of the target cell will be an important determinant of the dosing interval.

Pharmacokinetic considerations

The half-life of unmodified siRNAs *in vivo* is short (seconds to minutes).[50] This is predominately due to their rapid elimination by kidney filtration because of their small size (~7 kDa). They also can be degraded by endogenous serum RNases with a serum half-life of ~5–60 min. The circulating siRNA half-life can be extended to days by complexing the siRNAs with other molecules or incorporating them into various types of particles (to bypass renal filtration)[50–52] and by chemically modifying the sugar backbone[51,53–58] and capping the ends of the siRNA[50,55,58–60] to make them resistant to RNase digestion. Experience with developing oligonucleotide and ribozyme therapies has been used to develop chemically modified siRNAs with improved resistance to endogenous nucleases.[50,51,54,55,58,61] The backbone ribose of some residues is generally modified at the 2′ position to 2′-deoxyribose or 2′-*O*-methyl or 2′-fluoro substitutions.[51,53–58] Although, the siRNA half-life in serum can be extended from minutes to days by chemical modifications, modifying siRNAs often comes at the cost of less-efficient silencing. The impact of the modification on silencing may depend on its position in the siRNA sequence, whether it occurs on the sense or antisense strand, and the particular residue that is altered. Similarly, chemical modifications can greatly increase the *in vivo* siRNA half-life. Because renal clearance is faster than the rate of siRNA degradation, the increase in the *in vivo* half-life is most pronounced when siRNAs are delivered in a way that bypasses glomerular filtration (e.g. by producing serum complexes when siRNAs conjugated to cholesterol[50] bind to serum albumin or by incorporating siRNAs into stable nucleic acid-lipid particles[62]). Much of the experimentation that has been done to design the optimal strategy for balancing the opposing considerations of half-life and potency is unpublished. Moreover, what modifications are optimal will likely depend on the clinical indication and the strategy used to deliver the siRNA, as incorporation into complexes and particles may variably protect the siRNA from exposure to endogenous nucleases. It is noteworthy that many of the *in vivo* studies that have shown disease protection used unmodified siRNAs that were not optimized for half-life.[63–67]

Local versus systemic delivery

Although siRNAs are readily taken up into worm and fly cells,[68,69] most mammalian cells do not efficiently internalize these small molecules. This is even true of cells, such as dendritic cells and macrophages, that are actively sampling their environment.[44,70] Therefore, the major obstacle for using small RNAs as drugs is to deliver them into the cytoplasm of cells. An exception may be mucosal tissues. In the lung and vagina, siRNA uptake is extremely efficient and occurs even in the absence of transfection reagents.[42] For clinical indications where siRNAs only need to be delivered to a localized region, such as the eye,[71,72] pulmonary[73] or vaginal mucosa,[74] or superficial tumors,[75–78] efficient siRNA delivery and silencing can be achieved by mixing siRNAs with cationic lipid transfection reagents used for *in vitro* transfection and directly injecting the siRNA-lipid complexes into the relevant tissue or instilling it into the body cavity. A similar approach is certain to apply to the skin. Mixing siRNAs with other molecules known to carry nucleic acids into cells, such as certain cationic peptides, might also be used effectively for local delivery.[79,80] However, some cell types, such as lymphocytes, dendritic cells and hematopoietic stem cells, are refractory to transfection using cationic lipids. Therefore, even when these targets might be localized (e.g. in a joint inflamed by an autoimmune process), alternate delivery strategies may be needed.

The first demonstrations of the therapeutic potential of siRNAs for silencing disease-related genes delivered siRNAs systemically by rapid high-pressure intravenous injection ('hydrodynamic delivery'). This method leads to transient right-sided heart failure, where elevated venous pressures somehow enable siRNAs to get into cells in highly vascularized organs like the liver, pancreas and lungs.[44] Nonetheless, this strategy is too risky for human use. It is however possible to deliver siRNAs into an organ, such as the kidney, by rapid retrograde injection via catheter into the draining vein.[81] It may be possible to use hydrodynamic injection into a peripheral vein to treat skeletal muscle by blocking venous outflow using a tourniquet.[82] Elevated venous pressures are generated only in the targeted tissue without inducing potentially fatal heart failure. However, a minimally invasive method for delivering siRNAs requires alternate approaches. A variety of strategies that involve complexing siRNAs to cationic polymers or peptides or incorporating siRNAs into nanoparticles or liposomes have been proposed.[62,72,76,77,79,80,83] Alternately, siRNAs can be covalently or noncovalently linked to antibody fragments or ligands to cell surface receptors to limit the delivery of the siRNAs to cells that bear the specific receptor. These strategies probably deliver siRNAs via receptor-mediated endocytosis, although the trafficking of siRNAs into and within cells has not been well studied. The directed delivery of siRNAs into specific cells will decrease the amount of siRNAs needed for the efficient silencing of gene expression in the target organ or tissue and will reduce potential toxicity by preventing targeting of unintended cells and tissues. The optimal delivery strategy may differ between different therapeutic indications and will depend on efficiency and duration of delivery and silencing, lack of systemic toxicity, and lack of immunoreactivity, which would interfere with repetitive treatments. The first examples of effective systemic delivery have only recently been described.[50,62,84]

Choice of genes and diseases to target

As all cells have the RNAi machinery and any gene is a potential target, any disease caused by or greatly exacerbated by the expression of a dominant gene can in principle be treated by RNAi. This means that the list of potential indications is long. Diseases that are intractable or poorly responsive to current therapy are high on everyone's list, especially cancer, neurodegenerative disease, viral infection, and macular degeneration, and these are the disease models that have been most studied so far.[8,85–88] Viral infections are particularly attractive as RNAi constitutes an important primitive

antiviral response in plants and lower organisms. However, for viral infection and cancer, the potential for genetic mutation to escape from RNAi needs to be taken into account. Targeting multiple genes at once is one approach. Another is to target essential genes or highly conserved sequences whose mutation would come at a high cost for viral fitness or tumor-cell survival. For viruses, targeting host genes required for viral replication in addition to viral genes is another approach to minimizing the chances of escape.

Although the focus of this review is on using RNAi as a small molecule drug, an increasingly important application of RNAi – to pinpoint important disease-related genes that would be useful targets for other small molecule drugs – deserves mention. By knocking down a gene *in vitro* or in transgenic mice, it is possible to determine how important the gene might be in the pathogenesis of a particular disease. 'Knockdown' mice, bearing a transgene encoding for a short hairpin RNA (shRNA) precursor of an siRNA that can be expressed constitutively or conditionally in certain tissues or at specific times, provide a relatively quick way to assess the role of that gene *in vivo*.[89,90] Moreover, libraries of shRNAs designed to silence a subset of mouse or human genes can be used either *in vitro* (or potentially *in vivo* in mice) to screen for potential drug targets, which if inhibited might ameliorate disease course.[91–93]

The remainder of this review will discuss *in vivo* studies that have locally or systemically introduced siRNAs to silence disease-related genes in small animals. As the obstacles for systemic delivery are greater, local and systemic studies are discussed separately.

Local siRNA therapy

Clinical situations where siRNAs would need to silence disease-related gene expression locally in easily accessible tissues provide some of the most readily testable opportunities for exploring RNAi-based drug therapy. Local siRNA administration has shown benefit in small animal models involving the lung, vagina, subcutaneous tissue, muscle, eye and central nervous system.

Lung

Successful silencing in the lung has been achieved by intranasal or intratracheal administration of siRNAs. Remarkably, pulmonary epithelial cells have even been transduced *in vivo* by siRNAs given without transfection reagents or other delivery molecules.[42,73] This suggests that the lung (and possibly other tissues) may have a means for siRNA uptake, not generally present in most mammalian cells. The transformed cell lines and primary hematopoietic cells commonly used for *in vitro* RNAi experiments require a transfection reagent or other mechanism for siRNA uptake. The first therapeutic benefit in the lung was demonstrated for influenza A infection. siRNAs directed against conserved influenza sequences when complexed with the polycation polyethyleneimine (PEI) could be delivered to the lung by intravenous low pressure injection and reduce viral titers by 1–2 logs when given either before or after infection.[83] A concurrent study that combined hydrodynamic injection of the same siRNAs and intranasal instillation of siRNA complexed to oligofectamine was able to protect mice from lethal challenge with several highly pathogenic stains of influenza A.[74] Neither study found any evidence of interferon induction induced by siRNA treatment *in vivo*. These experiments established the proof of principle for treating pulmonary infections with siRNAs, although the hydrodynamic injection method is unlikely to be suitable for clinical use and the PEI carrier may also have unacceptable toxicity. The therapeutic possibility of siRNA treatment in the lung was advanced significantly by the recent study of Bitko *et al.*,[42] who were able to prevent and treat respiratory syncytial virus and parainfluenza virus, two significant pathogens in neonates that cause croup, pneumonia and bronchiolitis, by siRNAs directed against essential viral genes given intranasally with or without the transfection reagent Trans-IT TKO. Even in the absence of a transfection reagent, mice were protected from infection. Both viruses could be targeted simultaneously; however, if higher doses of one siRNA were used, protection against the second virus was not as efficient, suggesting possible competition for the RNAi machinery. In addition to lowering viral titers, clinical signs of infection including weight loss and increased respiratory rate were dramatically improved, as were laboratory indicators of disease severity such as leukotriene levels in bronchoalveolar lavage fluid and lung pathology. In another clinical setting, following traumatic hemorrhagic shock, the intratracheal instillation of siRNAs targeting proinflammatory chemokines, involved in the pathogenesis of acute lung injury, reduced chemokine production and consequent neutrophil infiltration in the lung.[95] This group also showed that intratracheal instillation only reduced gene expression in the lung, but not in more distal tissues, such as the liver. These recent studies suggest that intranasal delivery of chemically unmodified siRNAs to the lung should be an effective strategy for clinical use in the near future.

Vagina

Another mucosal surface, the vagina, has also recently been the target for RNAi-mediated silencing.[74] siRNAs mixed with Oligofectamine were efficiently taken up by epithelial and lamina propria cells throughout the vagina and ectocervix, but not in distal organs. Endogenous GFP expression in a fluorescent mouse in which every cell expresses GFP was silenced deep into the tissue. Moreover, silencing lasted for at least 9 days. By silencing viral genes, mice were protected from lethal herpes simplex virus 2 (HSV-2) infection when challenged intravaginally. Protection was even possible when siRNAs were not administered until 3 h after viral exposure. No toxicity from the siRNA-Oligofectamine complexes was found by histological examination for cell death or inflammatory infiltrates or by assaying for induction of interferon or interferon response genes by quantitative RT-PCR.

Subcutaneous tumor

Multiple studies have been able to inhibit the growth of subcutaneous tumors by injection of siRNAs targeting oncogenes or angiogenic genes when complexed with a variety of agents. In one study, *VEGF* siRNA mixed with proteolytically cleaved collagen (atelocollagen) was able to suppress the growth of the PC-3 prostate cancer cell

line in nude mice.[76] In another study, siRNAs directed against fibroblast growth factor were able to inhibit germ-cell tumor growth when mixed with either atelocollagen or constituted into liposomes.[77] Similarly intratumoral injection of liposomes containing *bcl-2* siRNA inhibited the outgrowth of subcutaneous PC-3 tumors.[96] Intraperitoneal administration of siRNAs directed against the growth factor receptor Her2/ErbB2 complexed with PEI efficiently targeted and inhibited a subcutaneous Her2+ ovarian tumor in nude mice.[97] More recently, intratumoral injection of a cocktail of siRNAs directed against *c-myc*, *MDM-2* and *VEGF* when mixed with an antibody fragment-protamine fusion protein was able to specifically target and inhibit subcutaneous B16 melanoma cells by directing the siRNAs only into the tumor cells via antibody binding to a cell surface receptor on the tumor.[84] These studies suggest the possible application of locally injected siRNAs as adjuvant therapy before or after surgical resection of tumors that have not yet metastasized. siRNAs might also be used to shrink a non-operable tumor to make it amenable to surgical removal. Topical application of siRNAs might also be highly effective for both benign and pre-maligant skin conditions, as well as malignant pigmented and non-pigmented skin cancers.

Muscle

Although multiple studies have used shRNA-encoding viral vectors to transduce muscle cells *in vivo*, only a few studies have delivered synthetic siRNAs into skeletal muscles either using electrical stimulation[98,99] or localized hydrodynamic injection,[82] however, none of these studies have used siRNAs to investigate siRNA therapy in a disease model. Electrical stimulation was used to deliver previously injected siRNAs into muscle cells to silence reporter genes, such as luciferase and eGFP or an endogenous gene (GAPD). This delivery method resembles electroporation, commonly used for *in vitro* transfection. It is not clear how far along a muscle, genes could be silenced; in one study images show silencing for at least 1 cm.[99] Silencing persisted without diminution for 11 days with some silencing still evident 23 days after treatment. Hydrodynamic injection of siRNAs into a peripheral vein of a mouse, rat or monkey limb that had been transiently isolated by reducing blood flow to and from the limb via a tourniquet led to the efficient silencing of a coinjected luciferase reporter plasmid.[82]

Eye

The eye was an early target for animal studies of siRNA-based therapy and is the target site of the first pilot clinical studies of siRNA-based therapy. Subretinal injection of siRNAs directed against VEGF complexed with a transfection lipid blocks the signals that induce neovascularization in the retina in response to laser photocoagulation damage, a model for age-related macular degeneration and other diseases, such as diabetic retinopathy, where blindness arises from hemorrhage of leaky new blood vessels.[72] Neovascularization in siRNA-treated eyes was only 25% of that in control eyes subjected to laser damage. Similarly, ocular neovascularization induced by proinflammatory CpG oligonucleotides or herpes simplex virus (HSV) infection was inhibited by injecting a cocktail of siRNAs targeting VEGF-A and two VEGF receptors without any delivery agent.[100] These same siRNAs could be delivered systemically in nanoparticles constructed with a polymer designed with PEI at one end, polyethylene glycol in the middle and an RGD peptide at the other end to direct binding to integrins on activated endothelial cells. In a disease model of post-surgical inflammation and fibrosis, subconjunctival injection of siRNAs targeting a TGF-β receptor complexed with lipids reduced inflammatory infiltrates and fibrosis. Such an approach might be useful to reduce postoperative scarring, although in practice, this therapy would have to balance the need for wound healing with the dangers of scarring. In yet another indication, siRNAs targeting c-jun and Apaf-1 were used to prevent degeneration of retinal ganglion cells following optic nerve transection.[101] Naked siRNAs directly injected into the optic stump after axotomy were taken up by some of the damaged neurons, allowing recovery from mechanical trauma. Whether such an approach could be developed to prevent permanent neuronal loss in other traumatic settings (such as spinal cord injury) remains to be seen.

Nervous system

Viral vectors encoding for shRNAs targeting mutated genes implicated in otherwise untreatable neurodegenerative diseases (SCA1 for spinocerebellar ataxia, SOD1 for amyotrophic lateral sclerosis) have shown therapeutic benefit in mice.[102–104] A few studies have also been able to demonstrate effective delivery and therapeutic effect of siRNAs injected into the central nervous system. Continuous intrathecal infusion by osmotic minipump in rats of stabilized siRNAs (0.4 mg/day) targeting a cation channel involved in sensing pain reduced the expression of the pain channel by 40% in dorsal root ganglia neurons and raised the threshold for pain responses in a model for chronic neuropathic pain induced by partial ligation of the sciatic nerve.[56] There was no overt inflammation from the treatment and siRNAs were more potent than antisense oligonucleotides targeting the same gene at alleviating pain in this model. Interestingly, the transduction of neuronal cells *in vivo* did not require a transfection reagent. Similarly, in mice stereotactic insertion of a cannula into the third ventricle for continuous infusion of stabilized siRNAs by minipump over 1–2 weeks was able to silence expression of a reporter EGFP gene as well as of endogenous dopamine and serotonin transporter genes at distal sites in the brain, again without any transfection reagent. Silencing was widespread, but variable in different regions of the brain and increased with the duration of the infusion. Changes in motor and behavioral activity were observed that were similar to those obtained by infusing specific inhibitors of the respective transporters. In another approach, localized transduction of neurons was achieved by electroporation in rats.[105] After siRNAs were stereotactically injected into the brain, the application of a mild electric current that did not on its own cause cellular apoptosis was able to reduce endogenous gene expression only in the area of the brain between the two electrodes. In addition, lipid complexes and immunoliposomes that have been used to deliver plasmid DNA encoding shRNAs might also be adapted to deliver siRNAs into brain cells.[106,107]

Systemic siRNA therapy

Although localized delivery of siRNAs to specific organs or tissues has been successful in an increasing number of experimental small animal models, this approach is limited to sites that are readily accessible. Delivery to deep-seated tissues and organs or to disseminated targets (i.e. lymphocytes or metastatic tumor cells) requires other strategies. One possible exception is treatment of parasitic infection. Parasites like other primitive organisms readily take up siRNAs in the absence of any transfection reagent.[108–110] Parasitic infections might therefore be readily treated by conventional intravenous administration of siRNAs silencing genes essential for the parasite to survive or replicate. Because siRNA uptake into mammalian cells is likely to be extremely inefficient and parasite genes are so distinct, toxicity is also likely to be minimal.

Hydrodynamic delivery

The first attempts at systemic delivery of siRNAs followed hydrodynamic transfection protocols previously used to deliver plasmid DNA into cells.[111,112] These involve the rapid intravenous injection (~5 s) of DNA or RNA in a large volume (typically 20–40% of the mouse circulating blood volume). This leads to uptake into liver, kidney, spleen, heart and lung cells. Hydrodynamic injection functions by creating a transient inversion of blood flow resulting from elevated venous pressures.[113] This purportedly promotes massive endocytosis. Most studies that used hydrodynamic injection focused on the liver because it is very efficiently targeted. Gene expression throughout the organ can be reduced by ~80%. In an early study, co-injection of a luciferase expression plasmid with an siRNA-targeting luciferase led to efficient suppression of luciferase expression in the liver and other central organs.[64,65,114] No long-term negative effects have been demonstrated after hydrodynamic injection, even after multiple injections, provided the animal survives the acute heart failure.[113] However, hydrodynamic injection requires a high level of technical skill and causes transient liver damage, characterized by cell swelling, some necrosis and modestly elevated serum liver transaminases. Moreover, it is difficult, if not impossible, to scale up for larger animals and is unlikely to be safe enough for human use. Therefore, the hydrodynamic injection studies of siRNA application to disease models should be interpreted as establishing proof of principle for human siRNA drug therapy, rather than as a potential mode of administration.

The susceptibility of the liver to a variety of agents that can induce acute or chronic liver injury including viral infection, autoimmune hepatitis, toxins, and liver transplantation, make it a good candidate for testing the therapeutic potential of siRNAs.[115–117] Many of these insults cause hepatic injury via engagement of the proapoptotic cell surface receptor Fas expressed on hepatocytes and upregulated in response to hepatic inflammation.[115,117] Fas-deficient (lpr) mice are protected from insults that would induce fulminant hepatitis in normal mice.[118,119] Hydrodynamic injection of labeled siRNAs showed that nearly 90% of hepatocytes took up the siRNAs, compared to ~40% of cells that could take up DNA vectors, making this approach extremely effective for siRNA delivery.[8,63] Injection of siRNAs targeting Fas led to degradation of Fas mRNA without affecting the expression of Fas-related genes, with the levels of Fas mRNA and protein being stably reduced by >80% for 10 days post transfection.[63] Importantly, this reduction of Fas had physiological consequences, preventing liver cell necrosis and inflammatory infiltration in concanavalin A-treated mice, a model for autoimmune hepatitis (Figure 1). In addition, Fas siRNA greatly increased survival in a fulminant hepatitis model induced by intraperitoneal injection of an agonistic Fas antibody. While all the control siRNA-treated mice died within three days, greater than 80% of the Fas siRNA-treated mice survived. Because Fas-mediated apoptosis plays a critical role in a wide variety of liver diseases, silencing of Fas by siRNA treatment may be of therapeutic value for preventing and treating acute and chronic liver injury induced by a range of insults. In fact, silencing caspase 8 (activated downstream after fas receptor engagement) has shown protection from both autoimmune and adenoviral hepatitis, and hydrodynamic injection of either Fas or caspase 8 siRNAs protected mice from sepsis in a bowel puncture model.[67,120]

Infection with hepatitis C virus (HCV) and hepatitis B virus (HBV) represents a major global health problem. These viruses infect ~270 and ~350 million people worldwide, respectively.[121–123] Although they are completely unrelated viruses, they both cause chronic hepatitis in a subset of infected individuals, which is associated with progression to cirrhosis and increased

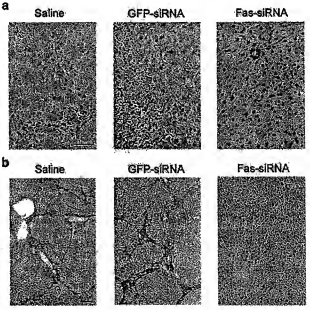

Figure 1 Fas gene silencing protects mice from fulminant hepatitis. (a) Concanavalin A was injected into mice as a model of acute autoimmune hepatitis. Mice were pretreated by hydrodynamic injection with saline or siRNAs directed against GFP or Fas. Representative liver histology is shown. This was the first demonstration of disease protection using siRNAs in an animal model. (Figure reprinted from Song *et al*.[63] with permission.) (b) In a chronic hepatitis model mice were injected every week with concanavalin A for 6 weeks and siRNA administration was deferred until after the second and fourth injections. Mice that received Fas siRNAs had reduced hepatic fibrosis at 7 weeks.

risk of hepatocellular carcinoma, with severely affected individuals requiring liver transplants. The similarity of symptoms is largely due to the liver damage associated with the immune response to these viruses, as neither virus is cytopathic. These viruses do not infect mouse or rat hepatocytes; as a consequence, no good small-animal model for these infections exists. Several groups have used hydrodynamic injection both to transduce mouse hepatocytes with viral replicons (cDNA versions of the viral genome) that then replicate the virus (but do not cause disease in mice)[124,125] and to deliver siRNAs designed to silence HCV and HBV gene expression and viral replication.[64–66] In the first demonstration of gene silencing in the liver, hydrodynamic injection of a DNA vector expressing an HCV NS5B protein fused to luciferase with an siRNA against NS5B led to a substantial inhibition of luciferase activity compared to a control siRNA.[64] Unmodified siRNAs targeting HBV were then shown to reduce viral replication in the mouse HBV replicon model.[65,126] More recently, hydrodynamic injection of certain chemically modified siRNAs at high concentrations (~50 mg/kg) was shown to have a more profound effect at inhibiting HBV replication than injection of unmodified siRNAs.[58] It was even possible to reduce HBV replication somewhat (~0.9 log) by repeated low pressure intravenous injections of 10–30 mg/kg modified siRNA directed against HBV, but six injections over 2 days were needed to achieve that modest effect. This approach was improved by incorporating the chemically modified, HBV-specific siRNA into a novel liposome complex forming a stable nucleic acid-lipid particle (SNALP).[62] The passive, intravenous administration of these particles efficiently reduced the level of serum HBV DNA in mice that had previously been injected with an HBV replicon. Incorporating siRNAs into these particles resulted in more sustained silencing and required lower and less-frequent doses of siRNAs than the hydrodynamic injection of chemically stabilized siRNAs.[62]

It is likely that other serious acute or chronic viral diseases involving internal organs can be controlled by administering siRNAs. Recently, hydrodynamic injection of siRNAs targeting Coxsackie virus B3, which causes acute and chronic viral myocarditis, was able to reduce viral titers in the heart and lung by at least 6 logs, and modestly prolonged survival in a highly susceptible mouse strain.[127]

Although systemic hydrodynamic injection is not practical for human use, it can potentially be used to inject the vein draining an organ, such as the kidney, to create localized elevated venous pressures that effectively deliver siRNAs. This was used to deliver Fas siRNAs into the kidneys to protect mice from acute tubular necrosis and death from renal ischemia-reperfusion injury.[61] Local instillation of siRNAs into the renal vein in a small clinically acceptable volume was as effective in this disease model as systemic hydrodynamic injection. However, approaches such as this require catheterization and therefore would be more costly and less practical for most indications than finding a method for peripheral intravenous administration.

Receptor-mediated systemic delivery

To use siRNAs as a systemic drug, more efficient delivery strategies are necessary to improve *in vivo* half-life, intracellular uptake and ideally target-specific cell-types. Several recent studies have begun to develop novel methods to enhance the potential drug-like properties of siRNAs and suggest that the siRNA delivery obstacle can be overcome. To this end, Soutschek *et al*.[50] conjugated cholesterol to the 3′-end of the target (antisense) strand of a chemically modified siRNA. Conjugation of siRNAs with cholesterol (Chol-siRNA) enabled the siRNAs to be taken up into cells via the ubiquitous LDL receptor. Intravenous injection of radiolabeled Chol-siRNA into rats showed an elimination half-life of 95 min compared to a 6-min elimination half-life for an unconjugated siRNA. This increased retention rate may be due to the propensity of cholesterol to bind serum albumin and be retained in the circulatory system. Injection of Chol-siRNAs targeting apolipoprotein B (ApoB), an essential protein for the formation of low-density lipoproteins (LDL), led to a significant reduction in *apoB* mRNA in the liver and jejunum, the primary sites of ApoB expression. This had a substantial effect on overall lipid metabolism: it decreased plasma ApoB by ~68% and decreased plasma LDL and cholesterol by ~40%. Although the conjugation of the siRNA with cholesterol promoted siRNA retention, the Chol-siRNAs did not discriminate into which tissues the siRNA would be delivered with labeled siRNAs being present in liver, heart, kidney, adipose and lung tissue, because the LDL receptor is ubiquitously expressed. However, the unconjugated control modified siRNAs were not detected in any of these organs. To obtain this impressive beneficial effect on serum cholesterol required a lot of siRNA (injections of 50 mg/kg on three consecutive days) and the effects were measured just 1 day later. There is no indication about how long the effect lasted. Nonetheless, this study led the way towards establishing a practical method for systemic intravenous injection of siRNA-based drugs.

Recently, a novel method for *in vivo* delivery of siRNAs to specific cell types was developed that takes advantage of the nucleic-acid binding properties of protamine, which nucleates DNA in sperm, and the specificity of fragment antibodies (Fab).[84] A protamine-antibody fusion protein (F105-P) was developed containing the protamine coding sequence linked to the C-terminus of the heavy-chain Fab fragment against the HIV-1 envelope protein. When mixed with siRNAs, F105-P delivered siRNAs and induced silencing only in cells expressing the HIV envelope. Moreover, the silencing was highly efficient and as effective as transfection *in vitro*. Using this system, siRNAs targeting the HIV-1 capsid protein inhibited HIV replication in hard-to-transfect HIV-infected primary T cells. *In vivo*, intratumoral or intravenous injection of mice with F105-P-complexed siRNAs specifically delivered fluorescently labeled siRNAs into HIV envelope-expressing B16 melanoma cells, but not into normal tissue or envelope-negative B16 cells (Figure 2). The antibody-mediated delivery of a cocktail of siRNAs (totaling ~3 mg/kg) targeting several genes involved in tumorigenesis by intratumoral or intravenous injection inhibited envelope-expressing tumor growth but not the growth of melanoma cells not expressing HIV envelope. In this study, the siRNAs were not chemically modified and were not optimized for improved pharmacokinetics. Cell-specific delivery and silencing were also obtained

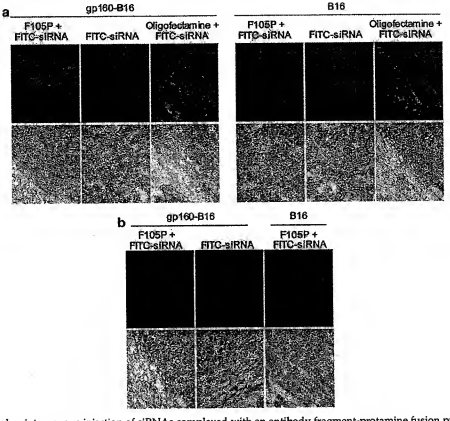

Figure 2 Intratumoral or intravenous injection of siRNAs complexed with an antibody fragment-protamine fusion protein delivers siRNAs only into mouse melanoma tumors expressing the cell surface receptor recognized by the antibody. (a) Fluorescent siRNAs, either naked or complexed with an anti-env-protamine fusion protein (F105-P) or oligofectamine, were injected into subcutaneous B16 melanomas expressing HIV env (left) or not (right). (b) Alternatively, F105-P loaded with fluorescent siRNA was injected intravenously. The tumors were harvested 12 h later for fluorescence microscopy (upper row) and hematoxylin and eosin staining (lower row). *In vivo* F105-P specifically delivers FITC-siRNA only into gp160-B16 tumors, but not into surrounding normal tissue or B16 tumors lacking *env*, while oligofectamine delivers FITC-siRNA into both tumor and neighboring tissues. Naked siRNAs do not efficiently get into any cells. Intratumoral injection is more efficient than intravenous injection. (Figure reprinted from Song *et al*.[84] with permission.)

with a single-chain antibody fusion protein that targeted ErbB2+breast cancer cells, demonstrating that this approach can be generalized to target other cell surface receptors. Because the siRNAs were not covalently linked to the antibody-protamine fusion protein, different siRNAs could be delivered with the same reagent. These results demonstrated that siRNA can be delivered systemically and target only cells expressing a specific cell-surface protein. Cell-specific targeting may reduce both the amounts of siRNA needed for therapeutic benefit as well as potential drug toxicity.

These examples are probably only the first of many strategies that are likely to be developed for systemic RNAi using siRNA-based drugs. Other methods used to deliver DNA plasmids for gene therapy, such as liposomes and immunoliposomes, which have been adapted to deliver plasmids encoding for shRNA precursors of siRNAs, could also deliver siRNAs or small siRNA precursors. In one study of mouse liver metastatic lung cancer, repeated injections of liposomes containing siRNAs directed against bcl-2 (10 mg/kg given 10 times) were able to inhibit tumor growth.[95] Similarly, nanoparticles containing cell targeting molecules on their outside and siRNAs within are also being developed.[52,100,128]

Conclusion

Just 3 years after RNAi was shown to work in mammalian cells, the first Phase 1 clinical studies targeting the VEGF angiogenic pathway in age-related macular degeneration have already begun. The interim analysis of one such study conducted by Sirna Therapeutics recently showed no evidence of clinical toxicity or disease progression in a small cohort. In the next few years it should become clear whether the promise of siRNA drug therapy to silence disease-causing genes with specificity and without undue toxicity will be realized. It should be an exciting time for researchers seeking to harness this powerful endogenous pathway to treat human disease.

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